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Vanessa Rédou

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Vanessa Rédou
Préparée au Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (EA3882)

COMMUNAUTES FONGIQUES DES SEDIMENTS MARINS DE SUBSURFACE : DIVERSITE, ORIGINE ET ROLE ECOLOGIQUE

Thèse soutenue le 27 octobre 2014
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Liste des abréviations

ADN : Acide désoxyribonucléique

ADNc : ADN complémentaire

ARN : Acide ribonucléique

ARNr : ARN ribosomique

ARNr 16S : petite sous-unité 16S de l'ARNr

ARNr 18S : petite sous-unité 18S de l'ARNr

ARNm : ARN messager

ATP : Adénosine triphosphate

CARD-FISH : *CAtalyzed Reporter Deposition-FISH*

CDH : Cellobiose déshydrogénase

FISH : *Fluorescent In situ Hybridization*

IPL : *Intact Polar Lipids*

ITS : *Internal Transcribed Spacer*

MPa : Méga pascal

NGS : *Next Generation Sequencing*

OTU : *Operational Taxonomic Unit*

Pb : Paire de bases

PCR : *Polymerase Chain Reaction*

PDA : *Potato Dextrose Agar*

pH : Potentiel hydrogène

PMO : Polysaccharide mono-oxygenase

PPi : Pyrophosphate inorganique

Q-PCR : PCR quantitative

RNAseq : *RNA sequencing*

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Nos connaissances concernant le monde du vivant sont relativement récentes et restent en perpétuelle évolution. Divisés en deux règnes par Carl Linnaeus au 18ème siècle, les organismes vivants étaient soit des animaux, soit des plantes. Cette classification dichotomique est abandonnée au profit d'un système à 3 règnes (Haeckel, 1866), puis à 5 règnes sur la base des différences de nutrition des *Plantae* (autotrophes), *Animalia* (hétérotrophes) et *Fungi* (saprophytes) tout en considérant les *Monera* (procaryotes) et les *Protista* (eucaryotes unicellulaires) (Whittaker, 1969). L'intégration de la phylogénie moléculaire à la classification du vivant développée par Carl Woese et George Fox en 1977 (Woese et Fox, 1977) bouleversa la vision du monde du vivant avec le premier arbre phylogénétique universel du vivant (Figure 1). La comparaison de la séquence d'un gène codant la petite sous-unité ribosomique des organismes vivants (16S pour les procaryotes et 18S pour les eucaryotes) a permis de générer l'arbre phylogénétique universel du vivant composé de trois lignées majeures appelées domaines, *i.e. Bacteria, Archaea* et *Eukarya* (Figure 2).

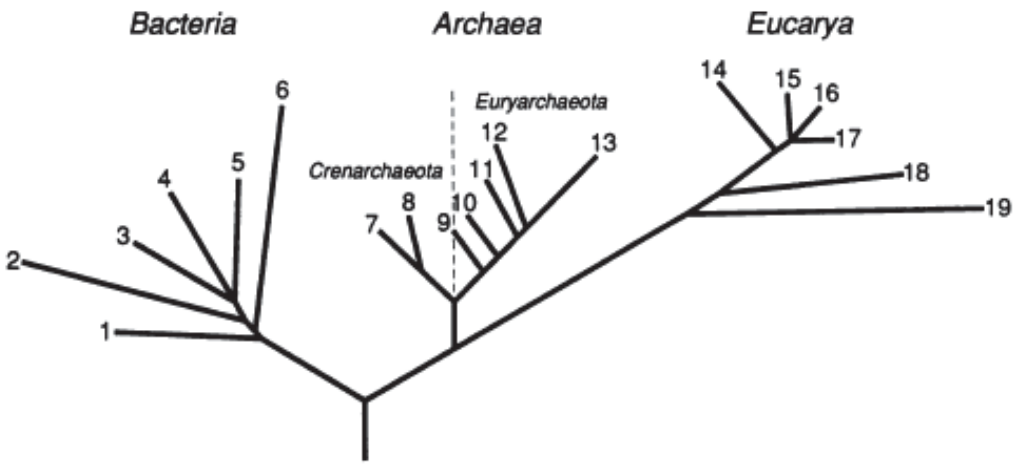


Figure 1 : Arbre universel du vivant selon Carl Woese *et al.* (1990)

Les chiffres sur les extrémités des branches correspondent aux groupes d'organismes suivants *Bacteria* : 1, Thermotogales ; 2, Flavobacteria ; 3, Cyanobactéries ; 4, bactéries pourpres ; 5, bactéries Gram-positives ; 6, bactéries vertes non sulfureuses. *Archaea* : 7, Pyrodictium ; 8, Thermoproteus ; 9, Thermococcales ; 10, Methanococcales ; 11, Methanobacteriales ; 12, Methanomicrobiales ; 13, halophiles extrêmes. *Eucarya* : 14, Animaux ; 15, Ciliés ; 16, Plantes vertes ; 17, Champignons ; 18, Flagellés ; 19, Microsporidies.

Les débats sur la représentation de la diversité des organismes et l'évolution du vivant sont en constante évolution, notamment avec les outils de séquençage haut-débit, mais encore aujourd'hui, les gènes codant pour l'ARNr 16S/18S sont des marqueurs moléculaires de référence pour identifier les organismes et pour représenter leurs relations de parenté. De récents travaux basés sur des analyses phylogénétiques multi-locus ont permis d'affiner la représentation du vivant. En comparant les gènes orthologues de 191 génomes complets, un « super-arbre » a permis de confirmer la classification du vivant en 3 domaines et d'affiner des positionnements phylogénétiques, notamment des lignées basales, *e.g. Firmicutes*, ou de poser des hypothèses sur la monophylie de certains groupes, *e.g. Acidobacteria* et sur la polyphilie de certains groupes reconnus comme monophylétiques, *e.g. Chroococcales* ou *Actinomycetales* (Ciccarelli *et al*., 2006).

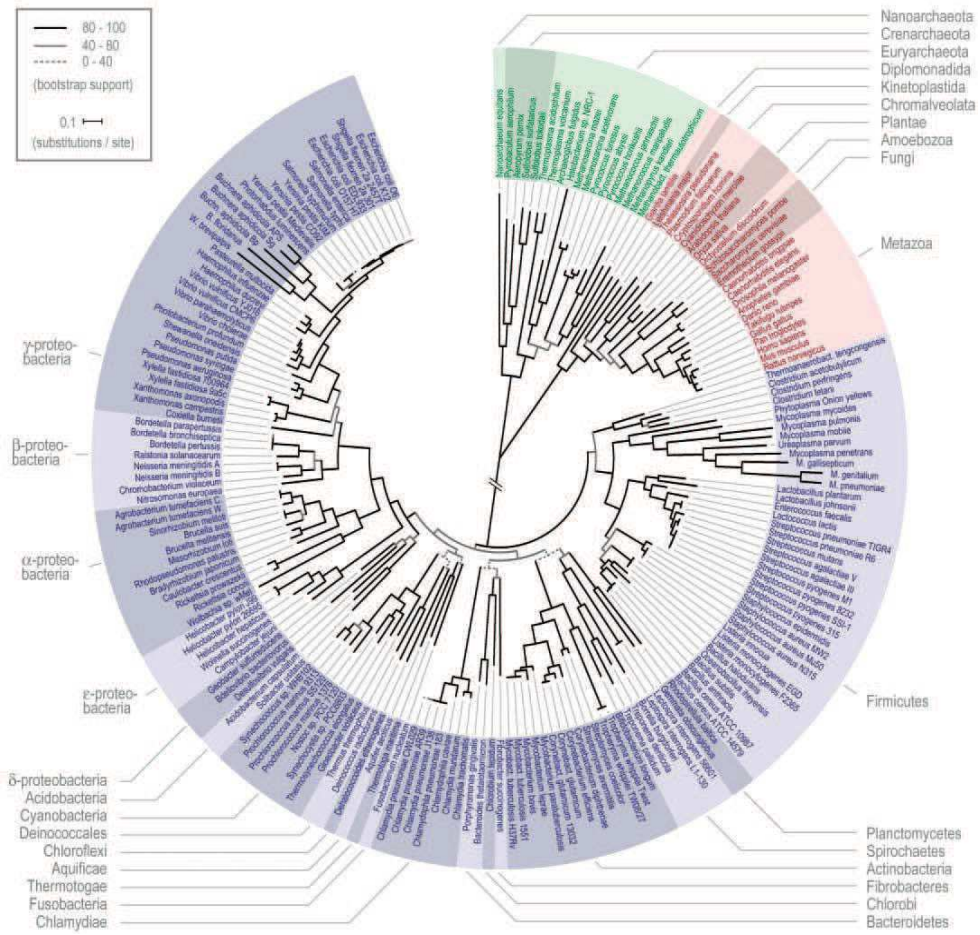


Figure 2 : Arbre phylogénétiques du vivant circulaire basé sur la comparaison de 31 gènes conservés de 191 espèces. D'après Ciccarelli *et al*. (2006)

En vert le domaine des *Archaea,* en rouge le domaine des *Eukarya* et en bleu celui des *Bacteria*.

L'arbre phylogénétique du domaine des eucaryotes comporte 8 règnes dont celui des Opisthokontes (Figure 3). C'est à ce règne qu'appartiennent les champignons qui représentent l'un des plus grands groupes d'organismes sur Terre en termes de nombre d'espèces (Schmit et Mueller, 2007). Bien que largement étudiés en milieu terrestre, les champignons sont capables de coloniser de nombreuses autres niches écologiques, qu'elles soient marines ou aquatiques, grâce à de grandes capacités d'adaptations aux contraintes environnementales.

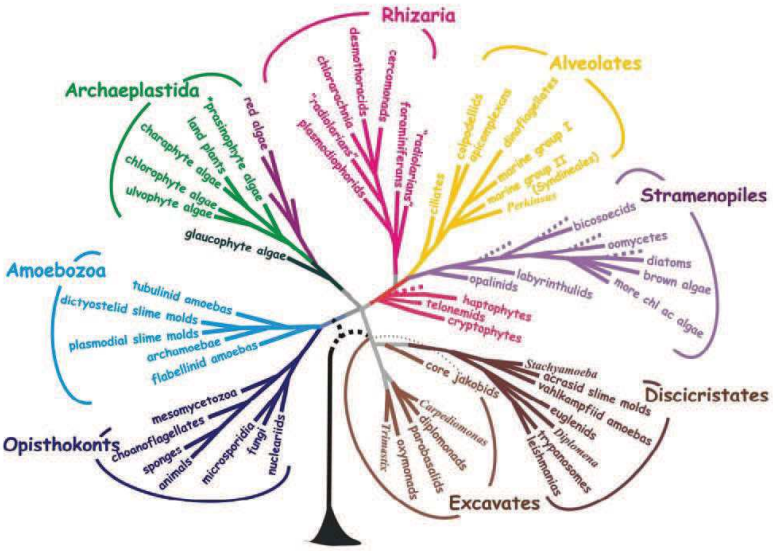


Figure 3 : Phylogénie des principaux groupes eucaryotes basée sur des données phylogénétiques et ultrastructurales. D'après Baldauf (2008)

L'objectif de cette introduction bibliographique est avant tout de présenter un état de l'art des connaissances sur les représentants du règne fongique, leurs adaptations aux contraintes environnementales et de présenter les données actuelles sur les champignons présents dans les écosystèmes sédimentaires marins. Une présentation des techniques utilisées pour caractériser les acteurs de la biosphère profonde sera également réalisée, en se concentrant sur les récentes techniques moléculaires de séquençage haut-débit.

1.1. Le règne fongique

1.1.1. Caractéristiques générales

1.1.1.1. Importance numérique des champignons

La diversité des champignons peut être représentée de 2 façons distinctes : (i) en fonction du nombre d'espèces décrites à ce jour et (ii) en fonction de l'estimation du nombre total d'espèces fongiques présentes dans l'ensemble de la biosphère terrestre.

(i) Par leur richesse en nombre d'espèces, les champignons représentent l'un des grands réservoirs de biodiversité sur Terre, juste derrière les insectes et plus conséquent que celui des bactéries (Figure 4).

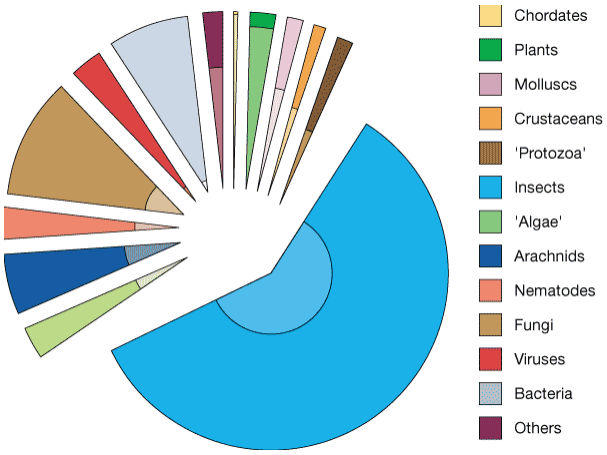


Figure 4 : Richesse des espèces dans les principaux groupes d'organismes vivants. D'après Purvis et Hector (2000)

Le nombre total d'espèces fongiques décrites fluctue entre 74 000 et 120 000 (Hawksworth, 2001; Kis-Papo, 2005). Les champignons semblent donc bien représenter l'un des groupes d'organismes les plus importants sur la planète (Hawksworth, 2001; Mueller *et al.*, 2004; O'Brien *et al.*, 2005; Hibbett *et al.*, 2007).

(ii) L'estimation du nombre total d'espèces fongiques présentes sur Terre a fait l'objet de nombreux débats avec des chiffres allant de 0,5 million (May, 1991), à 9,9 millions (Cannon, 1997) en passant par 1,5 million (Hawksworth, 1991, 2001). Cette dernière estimation est largement acceptée par la communauté scientifique. Plus récemment, un consensus a été obtenu concernant la limite inférieure de cette estimation. Il apparaît qu'un minimum d'environ 700 000 espèces serait présent sur Terre (Schmit et Mueller, 2007), ce qui positionnerait les connaissances actuelles sur la diversité spécifique des champignons à environ 1/10 de ce qui est réellement présent.

Les champignons représentent donc un réservoir de diversité, même si les connaissances actuelles sont loin d'être exhaustives. La biologie moléculaire et notamment la capacité des séquenceurs de 2ème génération (NGS) permettront (i) d'affiner l'estimation du nombre de champignons présents sur Terre, notamment avec les techniques de *metabarcoding* et (ii) d'augmenter le nombre de nouveaux taxons fongiques dans les collections de culture, notamment avec les techniques de métagénomique afin de mieux connaître les fonctions des champignons dans l'écosystème et adapter les techniques culturales pour les isoler.

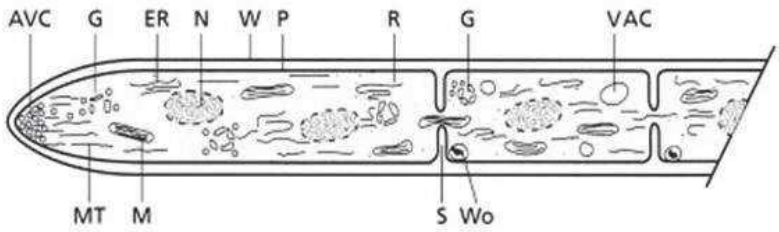
Si les champignons représentent, quantitativement, l'un des groupes eucaryotes les plus diversifiés, la diversité des champignons peut également être considérée en fonction du nombre de phylums fongiques. Avant de s'intéresser à la diversité taxonomique, la biologie de ces organismes sera tout d'abord précisée.

1.1.1.2. Structure

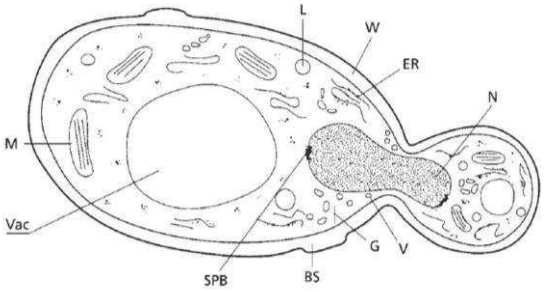
D'un point de vue structural, les champignons sont classés en deux catégories, la forme levure unicellulaire et la forme mycélienne pluricellulaire. Néanmoins, certaines levures et certains champignons filamenteux sont dimorphes et ont donc la capacité (i) de filamenter en formant des hyphes ou des pseudo-hyphes ou (ii) de passer d'une forme pluricellulaire filamenteuse à une forme levure bourgeonnante en fonction des conditions environnementales (absence d'oxygène, oligotrophie…).

La cellule fongique est constituée d'une paroi rigide, d'une membrane plasmique, d'un cytoplasme contenant divers organites (mitochondries, ribosomes, réticulum endoplasmique,

appareil de Golgi), de grandes vacuoles et un noyau, unique chez les levures. Le mycélium, chez les champignons filamenteux, est constitué d'hyphes soit cloisonnées, elles sont alors dites septées, soit non cloisonnées, elles sont alors dites coenocytiques.



(a)



(b)

Figure 5 : Structure de la paroi fongique chez (a) les champignons filamenteux et chez (b) les levures. D'après Deacon (2006)

BS = cicatrice d'un ancien bourgeonnement; ER = réticulum endoplasmique; G = appareil de Golgi; L = corps lipidique; M = mitochondrie; MT= microtubules; N= noyau; P = membrane; R = ribosomes; S = septum; SPB = corps polaire et fuseau; V = vésicule; Vac = vacuole; W = paroi; Wo = corps de Woronin.

Principalement composée de polysaccharides, la paroi fongique est organisée en microfibrilles de chitine (polymère de N-acétylglucosamine lié en β-1,4) associées à des polymères de glucoses (β-1,3 glucanes liés covalemment à des β-1,6 glucanes). L'ensemble de ces composants pariétaux est commun au règne fongique, mais chaque espèce peut avoir une proportion et une répartition différente de chaque élément. En effet, chez les champignons filamenteux, la chitine est le polymère majoritaire alors que le composant majeur de la paroi chez les levures est le glucane. La membrane plasmique est, quant à elle, essentiellement

composée de glycolipides spécifiques, appelés ergostérol, ce qui en fait une bonne cible antifongique (Figure 5).

1.1.1.3. Croissance

1.1.1.3.1. Champignons filamenteux

La croissance des champignons filamenteux est assurée par les hyphes et s'effectue au niveau de l'apex où la paroi est la plus fine (Deacon, 2006). La croissance peut être plus ou moins rapide selon les espèces et les conditions environnementales, de 0,9 mm/jour pour *Fusarium domesticum* à 10 mm/jour pour *Mucor circinelloides* sur mileu PDA à 25°C (K. Rigalma, UBO, communication personnelle). L'élongation apicale résulte d'un flux cytoplasmique de vésicules sécrétrices, orienté vers l'extrémité de l'hyphe. Ces vésicules forment un complexe dense, appelé Spitzenkörper ou corps apical, présent uniquement au niveau d'hyphes en croissance (Steinberg, 2007). Après fusion avec la membrane plasmique, ces vésicules sont libérées par exocytose et apportent les enzymes nécessaires à la lyse pariétale mais également les enzymes impliquées dans la synthèse pariétale, comme la chitine synthase et la glucane synthase (Steinberg, 2007). La clé de la croissance fongique réside donc à l'apex, au niveau du Spitzenkörper, où des activités enzymatiques antagonistes associées à une pression de turgescence élevée permettent la croissance des hyphes. Le septum, chez les champignons septés, est formé après élongation par constriction de la membrane plasmique. L'ensemble des hyphes, septés ou non, vont former le thalle aussi appelé mycélium (Deacon, 2006). Le mycélium a trois grandes fonctions : (i) la croissance, (ii) la sécrétion d'enzymes capable de dégrader la matière organique et (iii) l'absorption d'éléments nécessaire à la croissance et à la nutrition par endocytose.

1.1.1.3.2. Levures

Les levures sont des champignons unicellulaires qui se divisent pour la plupart par bourgeonnement ou par fission cellulaire (Mata et Nurse, 1998). Ces deux systèmes de division suivent la mitose mais la cellule fille issue du bourgeonnement est toujours plus

petite que la cellule mère alors que la fission cellulaire aboutit à deux cellules de taille identiques (Figure 6). Dans les deux cas, la présence d'un complexe protéique, le polarisome, est requis pour la polarisation des filaments d'actine vers le bourgeon. Cette polarité permet un transport vésiculaire vers le bourgeon, contenant les enzymes nécessaires au remodelage de la paroi dont la chitine synthase ainsi que de la membrane plasmique dont la glucane synthase. Les deux cellules se séparent par contraction au niveau de la jonction entre la cellule mère et la cellule fille par un anneau composé majoritairement de septine (Longtine et Bi, 2003).

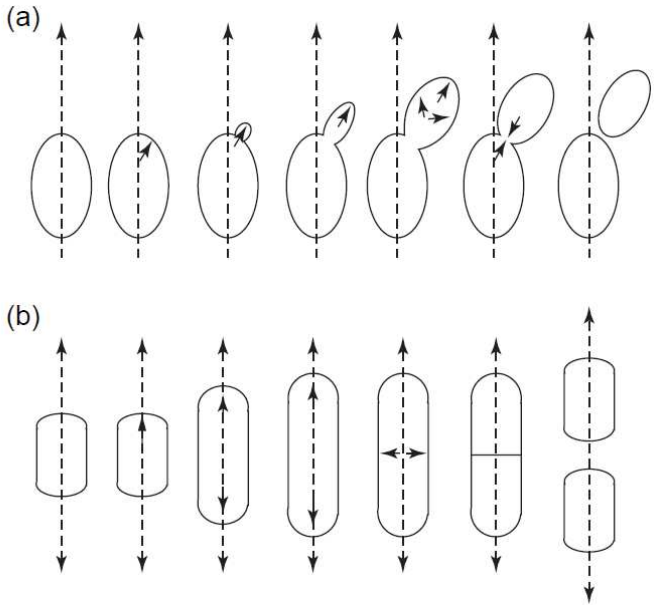


Figure 6 : Modes de croissance des levures par (a) bourgeonnement et par (b) fission. D'après Mata et Nurse (1998)

1.1.1.3.3. Dimorphisme

Selon les conditions environnementales, certaines espèces fongiques peuvent adopter les deux formes de croissance, levure ou filamenteuse. Ces levures sont alors caractérisées comme dimorphes. En effet, il a été montré que la levure *Candida albicans*, sous l'influence de stimuli environnementaux, pouvait opérer cette transition morphologique (Sudbery, 2011). Deux types de transitions sont observés, soit vers les hyphes, soit vers les pseudo-hyphes. Les

pseudo-hyphes sont morphologiquement distincts des hyphes car ils présentent une constriction au niveau des sites de cloisonnement et sont plus larges que les hyphes. En revanche, les hyphes forment de longs filaments réguliers septés (Figure 7).

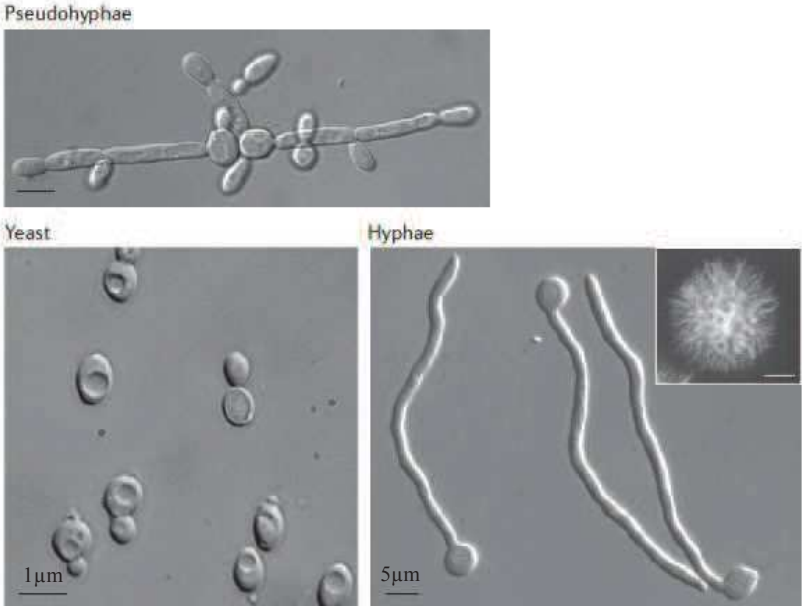


Figure 7 : Morphologies de la levure *Candida Albicans.* D'après Sudbery (2011)

A l'inverse, le champignon filamenteux *Mucor racemosus* présente une transition vers la forme levure. Un hexose fermentescible est toujours nécessaire pour la croissance sous forme levure et l'anaérobiose favorise en général la croissance sous la forme cellules bourgeonnantes, tandis que l'atmosphère aérobie induit généralement une croissance sous forme d'hyphes (Orlowski, 1991).

1.1.1.4. Reproduction

Les champignons peuvent se reproduire de façon sexuée ou asexuée. Le cycle sexué comprend trois phases : la plasmogamie, la caryogamie et la méiose. La fusion des cytoplasmes de deux cellules haploïde (plasmogamie) va former un mycélium secondaire,

dicaryotique. Les noyaux vont ensuite fusionner pour donner un noyau diploïde (caryogamie). Le noyau diploïde subit par la suite une méiose afin de rétablir l'haploïdie. La reproduction asexuée, ou multiplication végétative, se fait par division mitotique et aboutit à la formation de spores (Figure 8), dans certains cas flagellées.

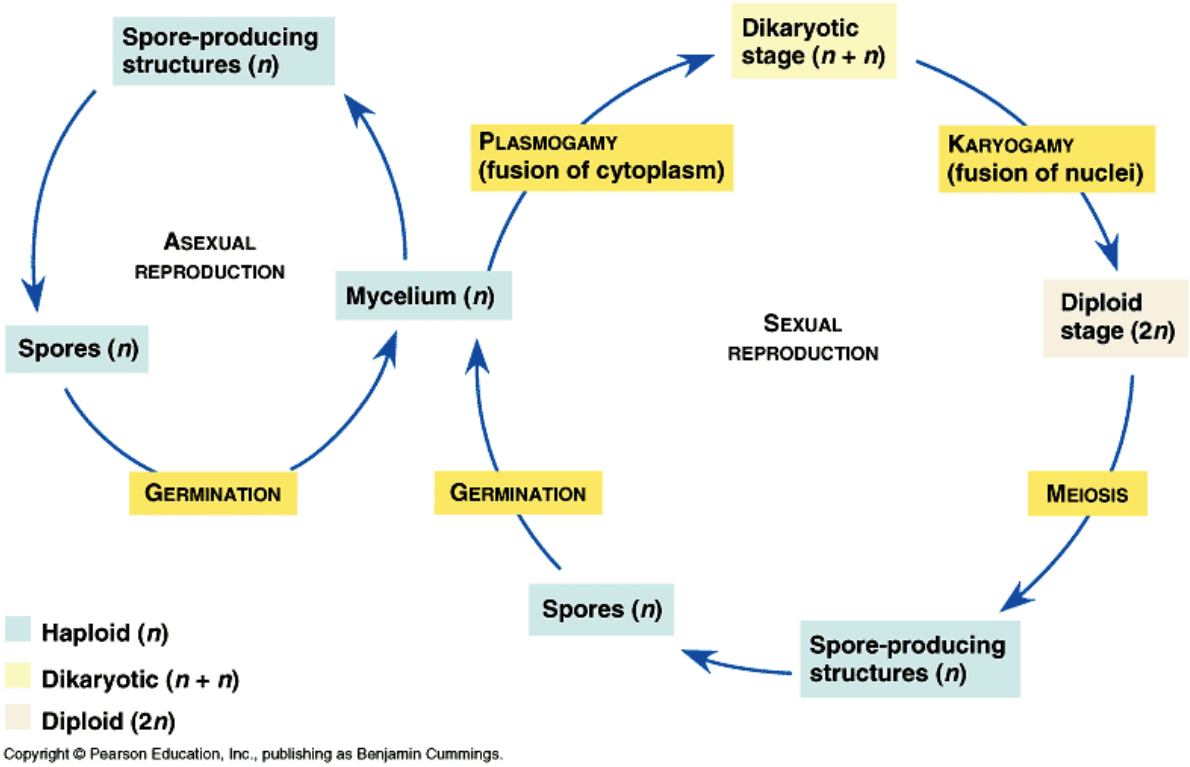


Figure 8 : Cycle de reproduction sexuée et asexuée chez les champignons

Les spores sont ensuite dispersées et véhiculées par l'eau ou l'air, qu'elles soient produites par fragmentation du mycélium, au sein de sporocystes (spores endogènes) ou bien portées par des cellules conidiogènes (spores exogènes). La dispersion des spores fongiques dans l'écosystème est principalement effectuée par le vent pour les champignons terrestres. Les spores vont s'immobiliser sous l'effet de la gravité puis sédimenter. En milieu humide, aquatique ou marin, les champignons produisent des spores avec une variété de formes et de tailles (Dang *et al*., 2007), reflétant ainsi leur adaptation à se disperser au sein de l'habitat. Majoritairement ces spores sont soit tétraradiées, ramifiées ou filiformes et dépassent 100 µm de longueur bien que de petites spores ovoïdes existent aussi. Ainsi, les différences de forme

des spores peuvent influencer le transport des champignons aquatiques et marins et leur capacité à coloniser la matière organique (Kearns et Bärlocher, 2008), en particulier dans l'enfouissement dans les sédiments. Les interactions entre la forme des spores et les propriétés des sédiments, notamment la porosité, peuvent influencer le transport et la colonisation des champignons dans les sédiments (Cornut *et al*., 2014). Les spores produites peuvent avoir un rôle dans la dispersion des champignons, mais peuvent également jouer un rôle dans la survie de l'organisme lorsque les conditions environnementales deviennent défavorables. En effet, lorsque les paramètres écologiques sont inadaptés à la croissance, ces spores sont capables de rester dans un état de dormance. Les mécanismes de dormance permettent aux spores de rester viables pendant une période prolongée dans l'attente de la disponibilité d'un substrat ou bien de conditions physico-chimiques favorables. Deux types de mécanismes de dormance sont reconnus, la dormance constitutive pour laquelle la germination est activée par des conditions particulières comme un choc thermique et la dormance exogène, plus commune, qui est imposée par des contraintes environnementales de température, d'oxygénation, d'activité de l'eau, de pH, *etc*. (Feofilova *et al*., 2012).

D'un point de vue taxonomique, les champignons sont classés selon leur mode de reproduction. En effet, le Code international de nomenclature pour les algues, les champignons et les plantes (CIN, Melbourne code, 2011), qui élabore les noms scientifiques attribués aux champignons, retient celui de la forme sexuée (téléomorphe). Cependant, de nombreux champignons se reproduisent uniquement de façon asexuée (anamorphe), le nom retenu est alors celui de la forme anamorphe. Les champignons pour lesquels on ne connait pas encore de forme téléomorphe ont été classés de façon artificielle dans un groupe appelé Deutéromycètes. Si historiquement un nom différent a été attribué pour la forme sexuée et asexuée, les règles de nomenclature ont vu plusieurs changements importants articulés autour du principe du "one fungus, one name", ainsi depuis le 1er janvier 2013, un seul nom s'applique à chaque espèce fongique (Hawksworth, 2011).

1.1.2. Diversité taxonomique

Sur la base d'analyses morphologiques et phylogénétiques, le règne des champignons a historiquement été divisé en 5 phyla : *Chytridiomycota*, *Zygomycota*, *Glomeromycota*,

Ascomycota et *Basidiomycota* (Shüβler *et al.,* 2001; James *et al.,* 2006). Si la monophylie des *Ascomycota*, *Basidiomycota* et des *Glomeromycota* a été confirmée par de nombreux travaux, les *Chytridiomycota* et *Zygomycota* sont quant à eux polyphylétiques (James *et al*., 2006; Bar-Hen *et al*., 2008). C'est d'ailleurs pour cette raison que l'étude de Hibbett *et al.* (2007), basée sur une analyse phylogénétique multi-locus, a remis en question la classification du règne des champignons à 5 phyla et proposé un règne à 9 phyla (Figure 9).

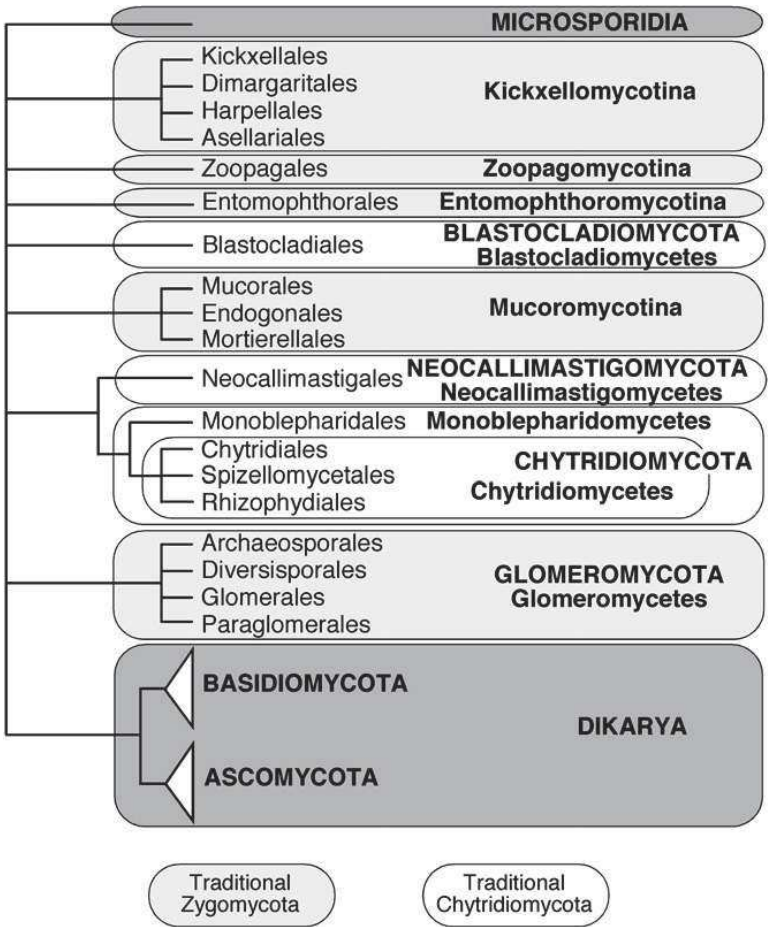


Figure 9 : Classification phylogénétique des champignons. D'après Hibbett *et al.* (2007)

Les changements majeurs dans la classification concernent les lignées basales, traditionnellement incluses parmi les *Chytridiomycota* et *Zygomycota*. Le phylum des *Chytridiomycota* est conservé mais restreint à la classe des Chytridiomycètes et Monoblepharidomycètes. Les *Blastocladiomycota* et les *Neocallimastigomycota*, auparavant

membre des *Chytridiomycota*, sont maintenant reconnus comme des phyla à part entière, ceux des champignons flagellés. Dans cette nouvelle classification, le phylum polyphylétique des *Zygomycota* disparaît et ses représentants sont répartis parmi les *Glomeromycota* et plusieurs sous-phyla *incertae sedis,* c'est à dire dont la classification n'est pas encore totalement définie, comprenant les *Mucoromycotina*, *Entomophthoromycotina*, *Kickxellomycotina* et *Zoopagomycotina* (Hibbett *et al*, 2007). Plus récemment, l'étude de Jones *et al.* (2011a) a permis l'identification et la validation (Jones *et al.*, 2011b) d'un nouveau phylum basal appelé *Cryptomycota* (*crypto*, caché ; *mycota*, règne des champignons) dont seul le genre *Rozella* est le représentant décrit (Figure 10). Ainsi, le positionnement phylogénétique et l'affiliation taxonomique des champignons sont évolutifs et la classification des champignons devrait évoluer en particulier grâce à la description constante de nouvelles espèces issues de niches écologiques inexplorées.

1.1.2.1. Les champignons zoosporiques

Les champignons zoosporiques sont des champignons unicellulaires qui ont la particularité de produire des spores uniflagellées, appelées zoospores. Considérés comme les lignées évolutives les plus anciennes du règne des champignons (James *et al,.* 2006), ils constituent moins de 1% des champignons décrits (Stajich *et al.,* 2009). Parmi les champignons zooporiques on retrouve 3 phyla, les *Blastocladiomycota*, les *Chytridiomycota* et les *Neocallimastigomycota.* Ce sont des champignons ubiquistes, retrouvés dans les milieux humides comme le sol, le milieu aquatique et également le milieu marin (Sparrow, 1960; Powell, 1993; Barr, 2001; Gleason *et al.,* 2011). Les champignons zoosporiques ont trois rôles majeurs au sein de ces écosystèmes, on les retrouve en tant que symbiotes, saprophytes ou parasites.

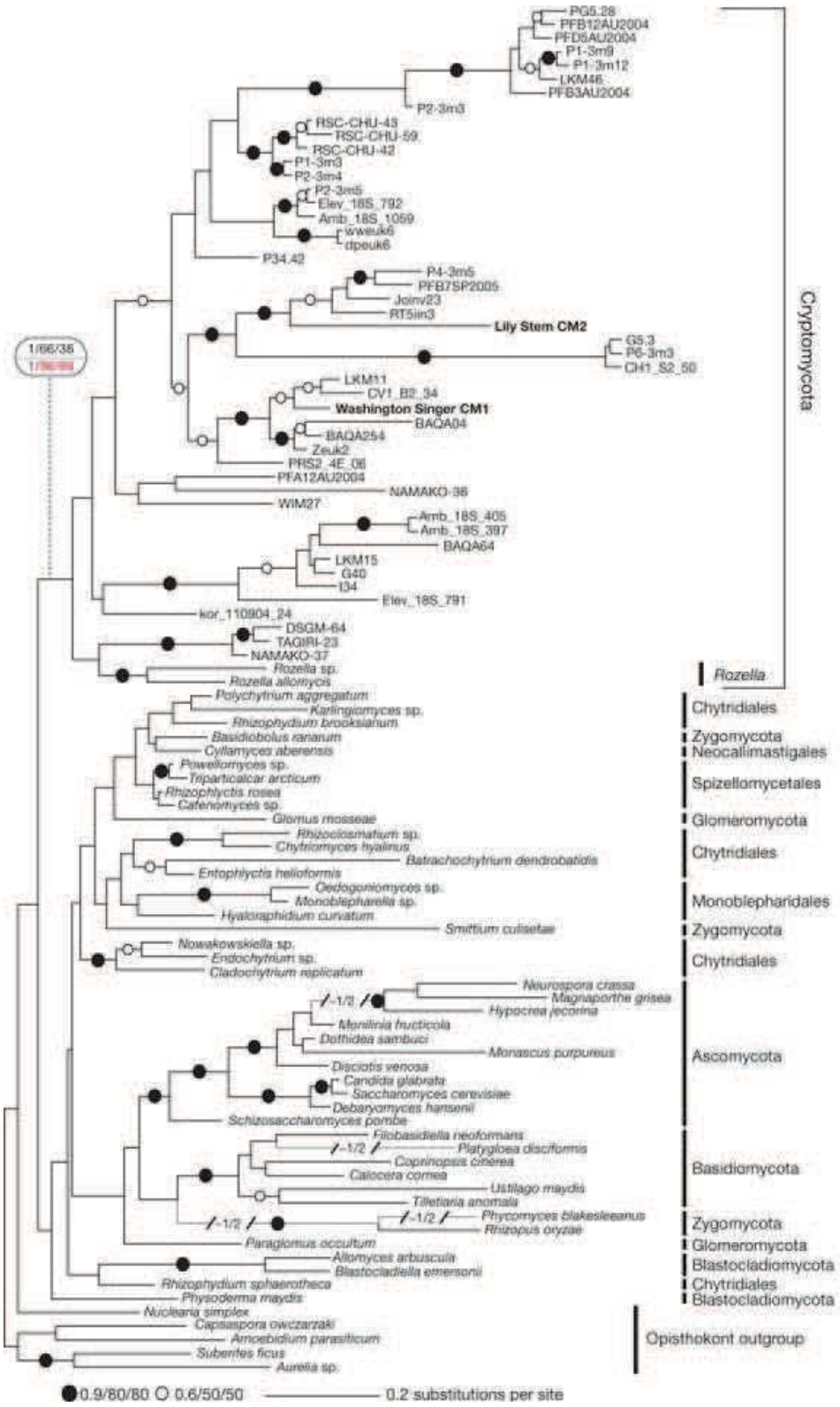


Figure 10 : Classification phylogénétique des champignons intégrant des séquences environnementales du clade des Cryptomycota. D'après Jones *et al.* (2011a)

Les *Neocallimastigomycota* sont des symbiotes généralement retrouvés au niveau du tube digestif des mammifères et des reptiles herbivores (Mackie *et al.,* 2004; Liggenstoffer *et al.*, 2010) tandis que les *Blastocladiomycota* et *Chytridiomycota* sont des saprophytes ou des parasites. Les champignons saprophytes sont des champignons qui dégradent la matière organique en décomposition. Les parasites quant à eux affectent un large éventail de macro/micro-organismes vivants comme le phytoplancton, le zooplancton, les macro-algues marines, les plantes supérieures, les animaux et autres champignons (Sparrow, 1960; Powell, 1993; Barr, 2001; Kagami *et al*., 2007). Ces parasites ont un impact écologique important sur les réseaux trophiques en raison de leur rôle dans la régulation naturelle de la taille des populations d'accueil, en particulier dans de nombreux écosystèmes d'eau douce (Kagami *et al*., 2007; Gleason *et al*., 2008). En effet, il a été montré dans une étude menée dans 14 lacs d'Amérique du Nord que le chytride *Polycaryum laeve*, hautement pathogène, peut causer une diminution drastique des populations de Daphnies, de petits crustacés (Johnson *et al.,* 2006). Compte tenu de l'importance des espèces de *Daphnia* comme brouteurs efficaces d'organismes phytoplanctoniques et d'autres composants de la boucle microbienne comme les bactéries, flagellés hétérotrophes ou ciliés, et comme une ressource alimentaire pour les poissons planctonophages, les chytrides peuvent affecter négativement la dynamique des populations et ainsi influencer de manière significative la globalité de la chaîne alimentaire.

1.1.2.2. Glomeromycota

Les champignons mycorhiziens à arbuscules forment un groupe monophylétique, le phylum des *Glomeromycota*. Moins de 200 espèces ont été décrites actuellement et un seul membre de ce phylum est connu pour former un type de symbiose différent, il s'agit de *Geosiphon pyriformis*, en s'associant non pas avec des photosymbiotes eucaryotes mais avec des photosybiontes procaryotes, des cyanobactéries (Redecker et Raab, 2006). Ce sont exclusivement des symbiotes obligatoires. En effet aucune culture de gloméromycète n'a été obtenue en l'absence de son symbionte. Tout comme les champignons zoosporiques, ils sont considérés comme primitifs en raison de l'absence de structures complexes comme les septums (hyphes coenocytiques). C'est parmi le phylum des *Glomeromycota* que l'on trouve les plus anciens fossiles de champignons. Les *Glomeromycota* auraient d'abord colonisé le

milieu aquatique en symbiose avec une cyanobactérie à l'image de *Geosiphon pyriformis* et ensuite colonisé les continents en contribuant directement au développement des végétaux en milieu terrestre (Shüβler *et al.,* 2001). Actuellement, on distingue quatre ordres, les Glomerales, les Diversisporales, les Paraglomerales et les Archaeosporales.

1.1.2.3. Dikarya

La majorité des espèces fongiques décrites (98%) sont membres du sous-embranchement des *Dikarya* qui comprend les deux phyla *Ascomycota* et *Basidiomycota* (James *et al*., 2006). Le terme *Dikarya* souligne la caractéristique de ces champignons qui est de posséder à une étape de leur cycle de vie deux noyaux dans une même cellule. Un des avantages de ce stade dicaryotique est l'augmentation de la diversité génétique des cellules filles à travers des phénomènes de recombinaison génétique (Stajich *et al.*, 2009).

1.1.2.3.1. Ascomycota

Les champignons affiliés au phylum des ascomycètes forment le groupe fongique le plus important avec près de 45 000 à 65 000 espèces d'écrites soit environ 65% de la diversité fongique (Taylor *et al*., 2004). Les ascomycètes capables de reproduction sexuée présentent une structure caractéristique en forme de sac, appelé asque, qui contient généralement 8 ascospores formées au terme de la méiose et de la mitose. Ces asques sont eux-mêmes regroupés dans une structure protectrice, appelée ascocarpe. Les ascomycètes qui suivent un cycle de reproduction asexuée produisent des conidies, issues d'un unique évènement de mitose, portées par une structure appelée conidiophore (Kendrick, 2000). D'un point de vue taxonomique, les ascomycètes sont répartis en 3 subphyla: *Taphrinomycotina*, *Saccharomycotina* et *Pezizomycotina*. Le sous-embranchement des *Pezizomycotina* est le plus important en termes de nombre d'espèces. Le sous-embranchement des *Saccharomycotina* contient une majorité de levures notamment utilisées en agro-alimentaire dont la plus connue est *Saccharomyces cerevisiae* communément appelée levure de boulangerie. On retrouve également des espèces parasites dont *Candida albicans*, un pathogène de l'homme. Le troisième sous-embranchement est celui des *Taphrinomycotina*, une lignée basale au sein des

ascomycètes avec à la fois des espèces à la forme levure et d'autres filamenteuses (Stajich *et al.,* 2009). D'un point de vue écologique, les ascomycètes sont des champignons ubiquistes retrouvés aussi bien au niveau des sols, des environnements aquatiques dulçaquicoles et marins. En milieu marin, ils sont notamment retrouvés en tant que saprophytes de matières végétales en décomposition ou en tant que parasites d'algues et d'animaux marins (Kohlmeyer et Kohlmeyer, 1979).

1.1.2.3.2. Basidiomycota

Les basidiomycètes représentent près de 35% des champignons décrits soit près de 22000 à 30000 espèces (Taylor *et al*., 2004). Les plus connus d'entre eux produisent les formes macroscopiques appelées champignons de forêts. La plupart des basidiomycètes présentent une structure caractéristique, nommée baside, qui contient les basidiospores formées au terme de la méiose (Kendrick, 2000). D'un point de vue taxonomique, les basidiomycètes sont répartis en 3 subphyla : *Pucciniomycotina*, *Ustilaginomycotina* et *Agaricomycotina*. Parmi le sous-embranchement des *Pucciniomycotina* on retrouve pour la plupart des espèces parasites obligatoires de plantes (responsables majoritaires de la rouille, maladie cryptogamique des végétaux), d'insectes et même de champignons. Le sous-embranchement des *Ustilaginomycotina* contient des parasites de plantes à l'exception de la levure *Malassezia*, associée principalement aux maladies de peau chez les animaux et les humains. Le troisième sous-embranchement, et le plus important quantitativement, est celui des *Agaricomycotina.* Parmi eux se distinguent les champignons comestibles tels que le champignon de Paris et d'autres qui peuvent être toxiques comme l'amanite phalloïde. On retrouve également des pathogènes de plantes et d'animaux. Les trois modes de croissance sont connus chez les basidiomycètes, la forme levure, la forme filamenteuse ou bien le dimorphisme (Stajich *et al.,* 2009). Bien que la majorité de ces micro-organismes soit retrouvée en milieu terrestre, certaines espèces, principalement des levures, sont retrouvées en milieu marin (Bass *et al.,* 2007).

1.1.3. Diversité métabolique et écologie

D'un point de vue métabolique, les champignons sont des organismes chimiohétérotrophes c'est-à-dire qu'ils utilisent des substrats organiques comme source d'énergie et de carbone. Pour assurer leur croissance, les champignons doivent utiliser des composés organiques issus de l'environnement. C'est donc en influant directement sur la chaîne alimentaire en tant que parasites, commensaux, mutualistes ou saprophytes, que les champignons ont un rôle clé dans le fonctionnement des cycles biogéochimiques.

1.1.3.1. Parasitisme

Les champignons peuvent tirer profit de la matière organique vivante. Ils sont alors considérés comme parasites et vivent aux dépens d'un être vivant. Souvent pathogènes, ils entraînent dans la majeure partie des cas la mort de leurs hôtes : bactéries, plantes, algues, d'autres champignons ou animaux (Lutzoni *et al.*, 2004). Reconnus comme ubiquistes, les parasites fongiques influencent la dynamique et la structure des populations (Hudson *et al.,* 2006; Miki *et al*., 2011). En milieu terrestre, on retrouve par exemple le chytride *Batrachochytrium dendrobatidis* parasite de batraciens (Longcore *et al*., 1999) ou bien encore *Olpidium brassicae*, parasite obligatoire de plantes (Hartwright *et al*., 2010). En milieu marin, les parasites fongiques peuvent vivre aux dépens d'algues comme *Chytridium polysiphoniae* (Küpper *et al*., 2006).

1.1.3.2. Commensalisme

Les champignons qui tirent profit de leurs hôtes sans leur nuire mais sans pour autant leur apporter un avantage sont appelés commensaux. Les champignons sont présents à l'état commensal principalement chez l'Homme. C'est le cas de la levure *Candida albicans*, commensale de l'intestin et des muqueuses (Ifrim *et al.,* 2013). Néanmoins, la limite peut être parfois étroite entre espèce commensale et espèce pathogène en cas par exemple

d'immunodéficience de l'hôte, témoignant d'une interaction dynamique et fluctuante (Iliev et Underhill, 2013).

1.1.3.3. Mutualisme

Si l'association apporte un avantage à chacun des deux acteurs on parle alors de mutualisme (symbiose au sens strict). Ces champignons vivent en symbiose avec des organismes autotrophes, au point que l'un ne peut vivre sans l'autre. En milieu terrestre, l'association entre les champignons et les racines des végétaux supérieurs est très répandue, on parle de mycorhizes. Il existe plusieurs types de relations mycorhiziennes. Les plus fréquentes sont les champignons mycorhiziens à arbuscules impliquant les champignons du phylum des *Glomeromycota* qui colonisent environ 80-90% des plantes vasculaires terrestres (Courty *et al*., 2010) et les ectomycorhizes comme par exemple l'ascomycète du genre *Tuber*, plus connu sous le nom de Truffe, en association avec des arbres comme le chêne ou le noisetier (Kües et Martin, 2011). Quelque soit la relation symbiotique, la plante contribue aux besoins carbonés du champignon et en retour le champignon satisfait les apports en nutriments minéraux de la plante tel que le phosphore ou l'azote, à partir du sol (Smith et Read, 2008). Le phosphore est souvent le facteur limitant pour la croissance des plantes car les phosphates issus du sol forment rapidement des complexes insolubles et de ce fait, il ne peut pas diffuser facilement jusqu'aux racines des plantes. Les champignons mycorhiziens aident à atténuer ce problème en fournissant un vaste réseau d'hyphes pour capturer des éléments minéraux et ainsi les transporter vers les racines des plantes (Deacon, 2006). Cette interaction est également retrouvée en milieu marin. Les champignons peuvent s'associer à des micro-organismes chlorophylliens comme les cyanobactéries ou les algues vertes. Le champignon, principalement des ascomycètes, fournit à l'algue protection, eau et sels minéraux et, en retour, celle-ci l'approvisionne en éléments carbonés produits grâce à la photosynthèse, on parle de lichens (Deacon, 2006).

1.1.3.4. Saprophytisme

Les champignons peuvent se nourrir de matière organique morte ou en décomposition telle que les feuilles mortes, les débris végétaux ou animaux, on les appelle alors des saprophytes. En effet, ils sécrètent une large gamme d'enzymes oxydatives et hydrolytiques capables de cliver des polymères complexes, tels que l'amidon, la cellulose, les protéines, la chitine, la kératine, et même les matières lignifiées plus complexes tels que le bois, en molécules plus simples, qu'ils peuvent ensuite assimiler (Peay *et al.,* 2008). Les champignons sont particulièrement important dans la décomposition de la cellulose, composant majoritaire de la paroi des végétaux et le polymère naturel le plus abondant sur Terre. De récentes études ont montrées que deux systèmes enzymatiques majeurs étaient impliqués dans la dégradation de la cellulose par les champignons : un système enzymatique classique, les cellulases (Baldrian et Valášková, 2008) et un système oxydatif faisant appel à la chimie de Fenton et aux monooxygénases de polysaccharides (PMO) (Žifčáková et Baldrian, 2012) (Figure 11). Les enzymes dégradant la cellulose, les cellulases, sont divisées en trois classes selon leur activité enzymatique : les endoglucanases, les cellobiohydrolases et les ß-glucosidases (Rabinovich *et al*., 2002; Baldrian et Valášková, 2008). Les endoglucanases hydrolysent les liaisons glycosidiques internes ß1-4, libérant ainsi de nouvelles chaînes de cellulose. Ces nouvelles extrémités vont servir de substrat aux cellobiohydrolases pour générer de la cellobiose. Finalement, les ß-glucosidases vont générer du glucose à partir des unités de cellobiose libérées précédemment. De plus, plusieurs systèmes oxydatifs où les enzymes n'interviennent pas directement sur la cellulose ont été proposés (i) les réactions catalysées par les PMO en collaboration avec les cellobioses deshydrogénases (CDH), (ii) un mécanisme basé sur la réduction de quinones en leurs hydroquinones et (iii) la production de radicaux hydroxyles catalysée par de petits glycopeptides sont impliquées dans la dégradation de plusieurs composants de la paroi cellulaire végétale, y compris la cellulose (Baldrian et Valášková, 2008).

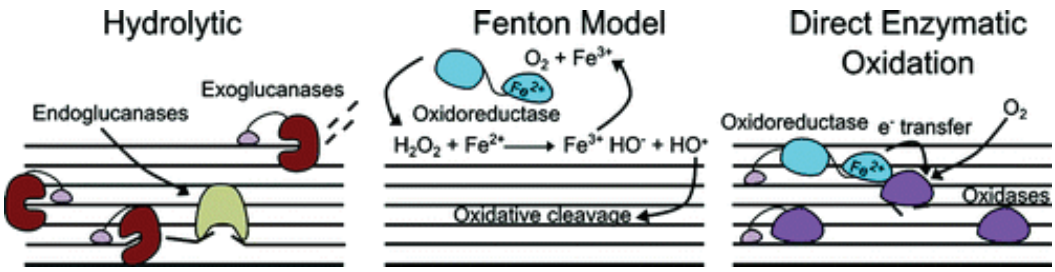


Figure 11 : Mécanismes hydrolytiques et oxydatifs proposés pour la dégradation de la cellulose. D'après Phillips *et al.* (2011)

(Gauche) Hydrolyse enzymatique : les cellulases agissent en synergie afin d'hydrolyser les liaisons osidiques; (Centre) Chimie de Fenton : les radicaux hydroxyles produits via la réaction de Fenton oxydent aléatoirement la cellulose; (Droite) Oxydation enzymatique : combinaison d'oxydases et d'oxydoréductases catalysant l'oxydation des liaisons osidiques.

En dégradant la matière organique morte, les champignons saprophytes remettent à la disposition des autres organismes des éléments minéraux essentiels de nouveau assimilables comme l'azote, le phosphore ou le carbone. Ils participent ainsi aux cycles biogéochimiques à travers le recyclage de la matière organique.

1.1.3.5. Rôle écologique

Les champignons sont des micro-organismes ubiquistes, capables de s'adapter à de nombreuses contraintes environnementales et qui ont donc pu coloniser de nombreux habitats. Historiquement uniquement rattachés aux environnements terrestres, les champignons sont également retrouvés en milieu aquatique dulçaquicole et marin.

En milieu terrestre, les champignons ont principalement été décrits comme étant saprophytes, jouant un rôle écologique important dans le recyclage d'éléments nutritifs à l'échelle planétaire. Essentiels à la croissance de 90% des plantes vasculaires, les champignons ont aussi un rôle majeur en temps que symbiotes dans l'écologie des sols (Smith et Read, 2008). C'est d'ailleurs une association symbiotique qui aurait permis aux plantes de coloniser le milieu terrestre (Simon *et al.*, 1993). Au-delà des écosystèmes terrestres, de récentes études ont mis en évidence que les écosystèmes marins abritent de nombreuses

niches écologiques encore peu étudiées et donc peu décrites pour les communautés fongiques, que ce soit au niveau de bois coulés (Barghoorn et Linder, 1944 ; Dupont *et al.,* 2009), de mangroves (Hyde et Lee, 1995; Alias et Jones, 2000; Sarma et Hyde, 2001; Arfi *et al*., 2012a, 2012b; Thatoi *et al*., 2013), de sources hydrothermales (Edgcomb *et al*., 2002; López-García *et al*., 2003, 2007; Burgaud *et al.,* 2009, 2010; Le Calvez *et al*., 2009), d'environnements anoxiques (Stoeck et Epstein 2003; Stoeck *et al.,* 2003, 2006, 2010; Takishita *et al.,* 2005; Jebaraj et Raghukumar, 2009; Jebaraj *et al.,* 2010), de bassins anoxiques hypersalés (Alexander *et al*., 2009; Stock *et al*., 2012) ou bien de zones de suintements froids (Takishita *et al*., 2007; Nagano *et al*., 2010; Nagahama *et al*., 2011). Les champignons marins mis en évidence à ce jour sont pour la plupart affiliés aux *Dikarya* et, en majorité, phylogénétiquement proches des champignons terrestres ce qui suggère que les champignons sont dotés d'une grande capacité d'adaptation aux conditions des environnements marins comme les fortes salinités, les faibles températures, la pression hydrostatique élevée et une relative oligotrophie. Au-delà de la colonne d'eau et couvrant plus de deux tiers de la surface de la Terre, une récente étude montre que les sédiments marins abriteraient près de 5,39 x 10^{29} cellules microbiennes (Parkes *et al*., 2014). Les communautés fongiques souterraines restent largement inexplorées mais les premières études ont révélées que malgré une faible diversité, les champignons sont parmi les micro-eucaryotes les plus abondants de l'écosystème sédimentaire et demeurent métaboliquement actifs jusqu'à 150 mètres sous la surface du plancher océanique (Edgcomb *et al.,* 2011; Orsi *et al*., 2013a, 2013b). Dominés par les basidiomycètes et principalement des levures (Bass *et al.,* 2007), les communautés fongiques des environnements marins profonds et de la biosphère profonde pourraient jouer un rôle essentiel dans la décomposition de composés complexes comme la matière organique réfractaire.

1.1.3.6. Ecophysiologie et adaptations

Comme pour tous les micro-organismes, la croissance fongique est influencée par différents paramètres environnementaux, physiques et chimiques tels que la température, le pH, l'activité de l'eau, l'oxygène ou encore la pression hydrostatique. Ces facteurs affectent non seulement le taux de croissance des champignons, mais peuvent aussi être responsable de

la mise en place de mécanismes d'adaptation. Dans cette partie, nous nous intéresserons à l'effet de 3 facteurs environnementaux caractérisant les environnements extrêmes et à l'adaptation aux conditions environnementales singulières.

1.1.3.6.1. Température

Les micro-organismes sont regroupés en cinq grandes catégories en fonction de leurs températures de croissance : les psychrophiles (littéralement « aimant le froid »), les psychrotrophes (qui tolèrent le froid), les mésophiles (qui se développent à des températures modérées), les thermophiles (littéralement « qui aiment la chaleur »; pour les procaryotes, il s'agit de ceux qui peuvent des développer à plus de 60°C), et les hyperthermophiles (qui se développent aux températures les plus élevées; pour les procaryotes, il s'agit uniquement de ceux dont la température optimale de croissance est supérieure à 80°C). La plupart des champignons sont mésophiles et seules quelques espèces fongiques peuvent se développer au-dessus de 37°C (Deacon, 2006) alors que de nombreux procaryotes peuvent se développer à cette température et même bien au-delà comme c'est le cas pour l'archée méthanogène hyperthermophile *Methanopyrus kandleri* capable de se développer jusqu'à 122°C (Takai *et al*., 2008). Le champignon le plus thermophile isolé à ce jour est *Thermomyces lanuginosus* avec une température limite supérieure de croissance d'environ 62°C (Deacon, 2006). A l'inverse, les champignons psychrophiles sont définis comme ayant un optimum de croissance d'environ 15°C (Robinson, 2001). De nombreux champignons sont capables de s'adapter aux environnements froids comme les régions polaires (Robinson, 2001) ou les fonds océaniques (Kohlmeyer et Kohlmeyer, 1979) qui ont une température stable de quelques degrés Celsius. Plusieurs champignons résistants au froid sont présents dans des environnements plus familiers comme par exemples les champignons du genre *Penicillium* qui se développent sur les denrées alimentaires dans les réfrigérateurs domestiques, à une température moyenne de 4°C (Deacon, 2006). Les mécanismes mis en œuvre pour s'adapter aux faibles températures font principalement intervenir une accumulation d'acides gras insaturés au niveau de la membrane pour en maintenr la fluidité, une accumulation de sucres tels que le tréhalose et de polyols tels que le glycérol au niveau du cytosol en réponse au stress thermique. Des protéines de choc thermique peuvent être synthétisées à des concentrations élevées en réponse à de

hautes températures. Ces protéines agissent comme des chaperonnes en assurant que les protéines de la cellule soient correctement repliées et que les protéines endommagées soient éliminées (Deacon, 2006).

1.1.3.6.2. Oxygène

Il est possible de classer les champignons en trois catégories en fonction de leurs relations à l'oxygène. La plupart des champignons sont aérobies stricts et utilisent l'oxygène comme accepteur final d'électrons au cours de la respiration. De nombreux champignons comme *Fusarium oxysporum, Mucor hiemalis* ou *Aspergillus fumigatus*, sont aérobies facultatifs c'est-à-dire qu'ils croissent en présence d'oxygène mais peuvent également se développer en son absence au cours de la fermentation des sucres. Le rendement énergétique à partir de la fermentation est très inférieur à celui de la respiration aérobie et la biomasse est souvent inférieure à 10% de celle d'une culture aérobie. Certains champignons sont quant à eux anaérobies stricts (Deacon, 2006). A l'heure actuelle, les seuls champignons anaérobies stricts connus sont affiliés au phylum des *Neocallimastigomycota* (Jebaraj *et al*., 2012). Ces organismes possèdent des organites particuliers appelés hydrogénosomes, fonctionnellement équivalents aux mitochondries des organismes aérobies, et sont impliqués dans la synthèse d'ATP par transfert d'électrons. Lorsque la croissance fongique utilise la cellulose, le glucose ou le fructose, les principaux sous-produits finaux libérés des hydrogénosomes sont le succinate, le lactate et l'éthanol (Marvin-Sikkema *et al.,* 1993).

1.1.3.6.3. Pression hydrostatique

Les micro-organismes sont regroupés en trois grandes catégories en fonction de leurs relations à la pression hydrostatique : les piezosensibles qui ne peuvent pas croître au-delà de 50 MPa, les piezotolérants qui ont un optimum de croissance à pression atmosphérique mais qui peuvent croitre au-delà de 50 MPa et les piezophiles qui montrent une meilleure croissance sous pression hydrostatique élevée (Yayanos, 1995). Avec une augmentation de 10 MPa/km à mesure que l'on s'immerge vers les abysses, la pression hydrostatique est un

paramètre important commun à différents écosystèmes marins (océan profond, zones de suintements froids, sources hydrothermales, sédiments profonds,…). Bien qu'aucun champignon piezophile n'ait encore été isolé d'environnements marins profonds, divers procaryotes l'ont été (Yayanos *et al*., 1979; Bale *et al*., 1997; Kato *et al*., 1998; Alain *et al*., 2002; Nogi *et al*., 2004; Wang *et al*., 2004; Takai *et al*., 2009; Birrien *et al*., 2011). Les études portant sur la croissance fongique sous pression hydrostatique ont mis en évidence une diversité de champignons filamenteux et de levures capables de croître dans ces conditions (Lorenz et Molitoris, 1997; Raghukumar et Raghukumar, 1998; Damare *et al*., 2006; Damare et Raghukumar, 2008; Singh *et al*., 2010). Même si la plupart des isolats tolèrent les hautes pressions, ils présentent généralement une meilleure croissance sous pression atmosphérique (Nagano et Nagahama, 2012). La levure *Saccharomyces cerevisiae* a été bien étudiée afin de mettre en évidence les mécanismes d'adaptation aux hautes pressions hydrostatiques (Shimada *et al.,* 1993; Abe et Horikoshi, 1995, 1998, 2000; Fernandes *et al*., 2004; Abe, 2007; Freitas *et al*., 2012). *S. cerevisiae*, qui est une levure piezotolérante, est capable de se développer à des pressions supérieures à 50 MPa (Fernandes *et al*., 2004). Ainsi, lorsque la pression hydrostatique augmente, les cellules de levure doivent rapidement s'adapter et des mécanismes de défense sont mis en œuvre pour surmonter les effets induits par la pression hydrostatique (Fernandes *et al*., 2004). Il a été montré que *S. cerevisiae* est capable de modifier la composition de sa membrane afin de tolérer les hautes pressions hydrostatiques en augmentant la proportion d'acides gras insaturés et d'ergostérol (Simonato *et al*. 2006). Cette stratégie adaptative vise à augmenter la fluidité membranaire pour contrebalancer l'effet rigidifiant de la pression hydrostatique et permettre ainsi de maintenir les fonctionnalités des membranes cellulaires.

Le but de cette partie était de montrer que les champignons sont des micro-organismes très diversifiés, de par leur diversité taxonomique, leur plasticité morphologique et leur capacité d'adaptation à de nombreuses contraintes environnementales. Le but était également de montrer que les champignons sont des organismes hétérotrophes et impliqués dans différents réseaux trophiques. Ils ont par conséquent un rôle clé à l'échelle de la biosphère terrestre via la dégradation et le recyclage de la matière organique, ainsi que dans la structure mais aussi dans la dynamique des populations.

1.2. Les sédiments marins : un écosystème complexe

Recouvrant près de deux tiers de la surface terrestre, les sédiments marins résultent de l'accumulation de matière organique et inorganique plongeant à travers la colonne d'eau et se déposant sur le plancher océanique au cours des temps géologiques (Henrichs, 1992). Les sédiments marins représentent ainsi le principal réservoir de carbone organique sur Terre (Fry *et al*., 2008). Considérés abiotiques il y a encore une vingtaine d'années, les sédiments marins représentent un vaste réservoir de biodiversité et sont désormais considérés comme une nouvelle dimension du vivant.

1.2.1. Contexte géologique

La croûte océanique, qui forme le plancher de l'océan profond, est fragmentée en plaques mobiles les unes par rapport aux autres. Constituée, principalement de roches basaltiques, la croûte océanique est formée de manière continue par l'activité volcanique et tectonique des dorsales océaniques (Jørgensen et Boetius, 2007) (Figure 12).

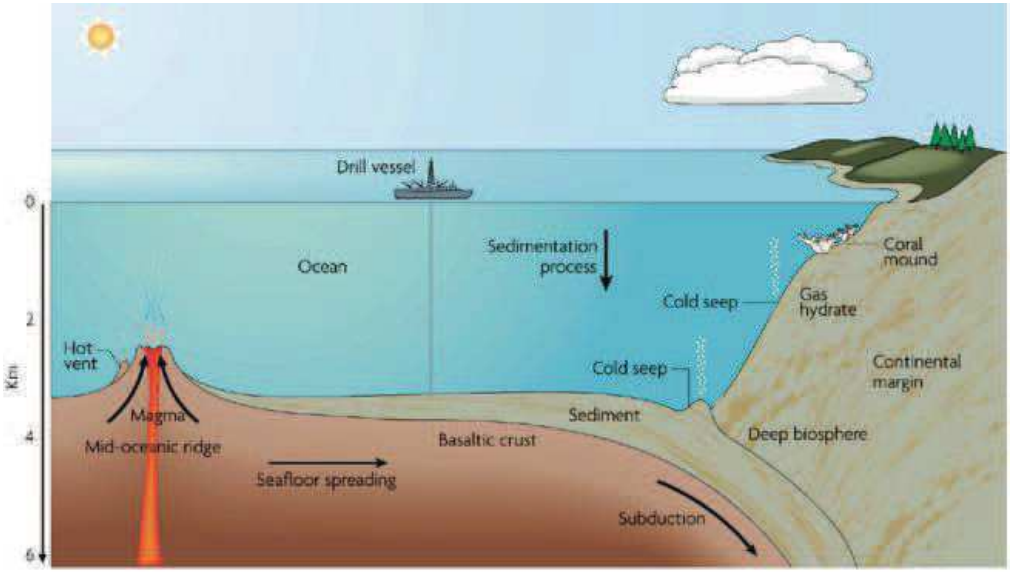


Figure 12 : Coupe transversale du plancher océanique montrant l'expansion des fonds océaniques. D'après Jørgensen et Boetius (2007)

Le plancher océanique est recouvert d'une couche de sédiments composés de particules d'origines biogènes, terrigènes, volcaniques et authigènes (minéraux ayant cristallisés au sein d'une roche sédimentaire au cours de la diagenèse) et d'épaisseur variable en fonction de la localisation (Jørgensen et Boetius, 2007; Fry *et al*., 2008). En effet, au niveau des zones côtières et des marges continentales, les apports importants de matière organique, principalement d'origine terrigène, engendrent une forte activité sédimentaire et peuvent atteindre des épaisseurs supérieures à 10 km (Schulz et Zabel, 2006). Les sédiments terrigènes, issus de l'érosion des roches et des sols, sont composés de sables, de limons et d'argiles déversés à la mer notamment par le ruissellement des rivières. Dans le domaine océanique, la sédimentation est moins intense et la teneur en matière organique y est plus faible, comme par exemple le gyre subtropical du Pacifique Sud qui présente un taux de sédimentation extrêmement bas (D'Hondt *et al*., 2009). Les sédiments de ces régions oligotrophes sont principalement composés d'argiles siliceuses (SiO_2) ou de carbonate de calcium ($CaCO_3$) d'origine biogène provenant principalement du phytoplancton marin (Dunne *et al*., 2007). Que ce soit au niveau des marges continentales ou bien dans le domaine océanique, la taille des particules, du micromètre pour les argiles au centimètre pour les graviers, influence notamment la porosité des sédiments et affectent la circulation des fluides et le transport des substrats accessibles aux micro-organismes (Orcutt *et al*., 2011).

1.2.2. Contexte biogéochimique

La majeure partie de la matière organique issue de la colonne d'eau est dégradée par les micro-organismes dans les premières couches sédimentaires. Seule une faible partie de cette matière organique résiduelle s'enfouit (moins de 1%) dans les sédiments marins profonds (Konhauser, 2009; Arndt et *al.,* 2013). Suite à un ensemble de processus physico-chimiques et biologiques que l'on appelle la diagénèse, la composition et la structure des sédiments sont modifiées et, de leur dépôt au niveau du plancher océanique jusqu'à leur enfouissement, ils sont convertis en roche sédimentaire (Zonneveld *et al*., 2010). Ainsi, les sédiments récents qui se déposent au cours du temps, compriment les sédiments plus anciens, diminuant leur porosité. Plus en profondeur, sous l'effet de la température et de la pression, la

matière organique subit une suite de transformations physico-chimiques et devient de plus en plus insoluble et réfractaire aux dégradations enzymatiques, aboutissant à la formation de géopolymères, appelés kerogènes (Zonneveld *et al*., 2010; Arndt et *al.,* 2013). Ainsi, la matière organique des sédiments marins profonds présente une biodisponibilité limitée et donc probablement insuffisante pour fournir l'énergie suffisante aux communautés de subsurface (Burdige, 2007). Cependant, les contraintes physiques en profondeur, comme la température et la pression, influencent les réactions de maturation de cette matière organique récalcitrante. Via des procédés tel que la condensation, les kérogènes abiotiques peuvent être convertit en hydrocarbures fossiles comme le pétrole et le gaz (Quigley et Mackenzie, 1988; De Leeuw et Largeau, 1993) potentiellement utilisables à travers différentes voies métaboliques aboutissant à la synthèse de méthane en fonction des conditions environnementales (Dolfing *et al*., 2008).

Outre les procédés physico-chimiques, la matière organique est également dégradée par des processus biologiques via les micro-organismes. La matière organique parvient à l'interface eau-sédiment majoritairement sous forme de macromolécules, telles que les polysaccharides et les protéines (matière organique particulaire) et ne peut donc pas être directement assimilée par les micro-organismes. Ces macromolécules sont dépolymérisées, sous l'action d'exoenzymes microbiennes hydrolitiques, en monomères de type sucres et acides aminés ou en produits finaux tel que le dioxyde de carbone, CO_2, et l'eau, H_2O (Wakeham et Canuel, 2006; Zonneveld *et al*., 2010) (Figure 13). Les monomères sont soit utilisés pour synthétiser des constituants cellulaires et dans ce cas réintroduits dans le cycle biologique, soit minéralisés lors de leur utilisation par les procaryotes comme source d'énergie (Burdige, 2007).

La présence d'oxydants forts, le taux de sédimentation et la taille des particules de la matière organique déterminent la succession des principaux métabolismes microbiens responsables de la dégradation de la matière organique dans les sédiments marins (Konhauser, 2009). En surface, l'oxygène est généralement rapidement consommé par les micro-organismes hétérotrophes capables de dégrader des composés organiques complexes à travers la respiration aérobie (Konhauser, 2009). A mesure que l'on s'enfonce dans le sédiment, la concentration en oxygène diminue et d'autres accepteurs d'électrons sont réduits séquentiellement comme les NO^{3-}, Mn^{4+}, Fe^{3+}, SO_4^{2-} et CO_2 au travers de la sulfato-réduction

et la méthanogénèse principalement, jusqu'à épuisement des substrats organiques labiles (DeLong, 2004; D'Hondt *et al*., 2004) (Figure 14). Ainsi, la composition des communautés et du sédiment change drastiquement au cours de l'enfouissement de la matière organique qui devient de plus en plus réfractaire sous l'accumulation de composés inorganiques tels que NH^{4+}, Mn^{2+}, Fe^{2+}, S^{2-} et CH_4 (Parkes *et al.,* 2007). Une partie de cette matière organique réfractaire peut être transférée jusqu'à la zone oxique par biodégradation et bioturbation de la zone anoxique.

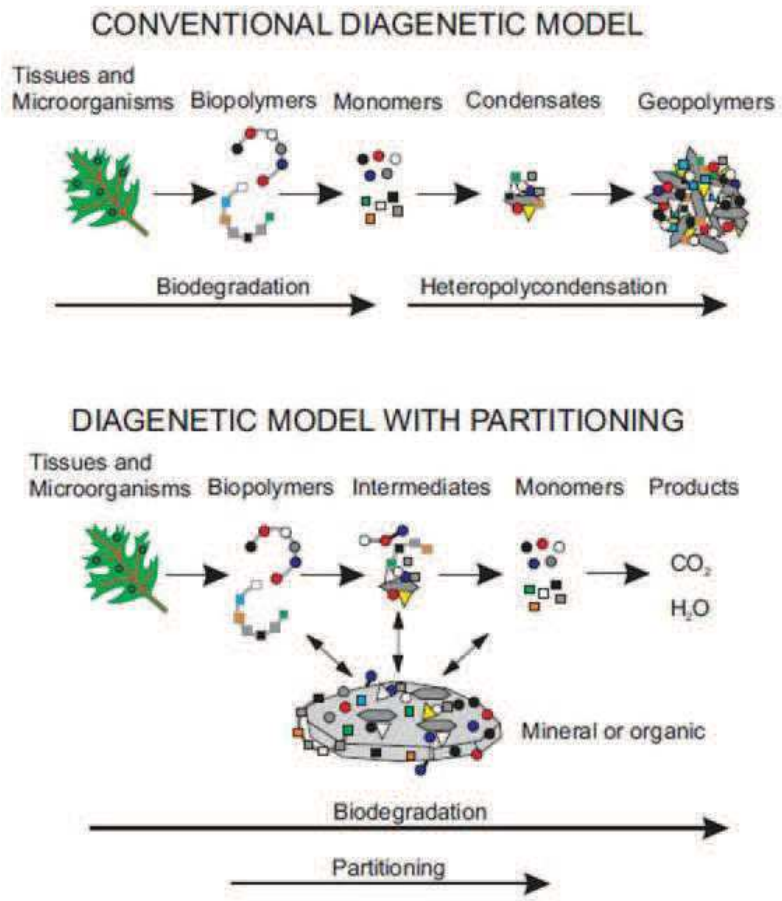


Figure 13 : Schéma global de la diagénèse. D'après Zonneveld *et al*. (2010)

Au cours du modèle classique de biodégradation/repolymérisation (schéma du haut), les produits de dégradation se combinent spontanément pour former des géopolymères. Au cours du modèle alternatif de biodégradation/sorption (schéma du bas), certains intermédiaires de la dégradation microbiennes échappent à l'absorption et sont dépolymérisées en monomères et en produits finaux.

Les micro-organismes responsables de la dégradation de la matière organique appartiennent aux procaryotes, bactéries et archées, mais aussi aux eucaryotes. Les eucaryotes occupent généralement les couches supérieures du sédiment mais sont aussi connus pour remettre en circulation la matière organique des régions anoxiques à la zone oxique par bioturbation (Zonneveld *et al*., 2010). Ainsi, le potentiel de reminéralisation de la matière organique via les micro-organismes dépend de la porosité des sédiments comme nous l'avons vu dans la partie précédente mais aussi de la disponibilité des accepteurs d'électrons présents dans l'environnement.

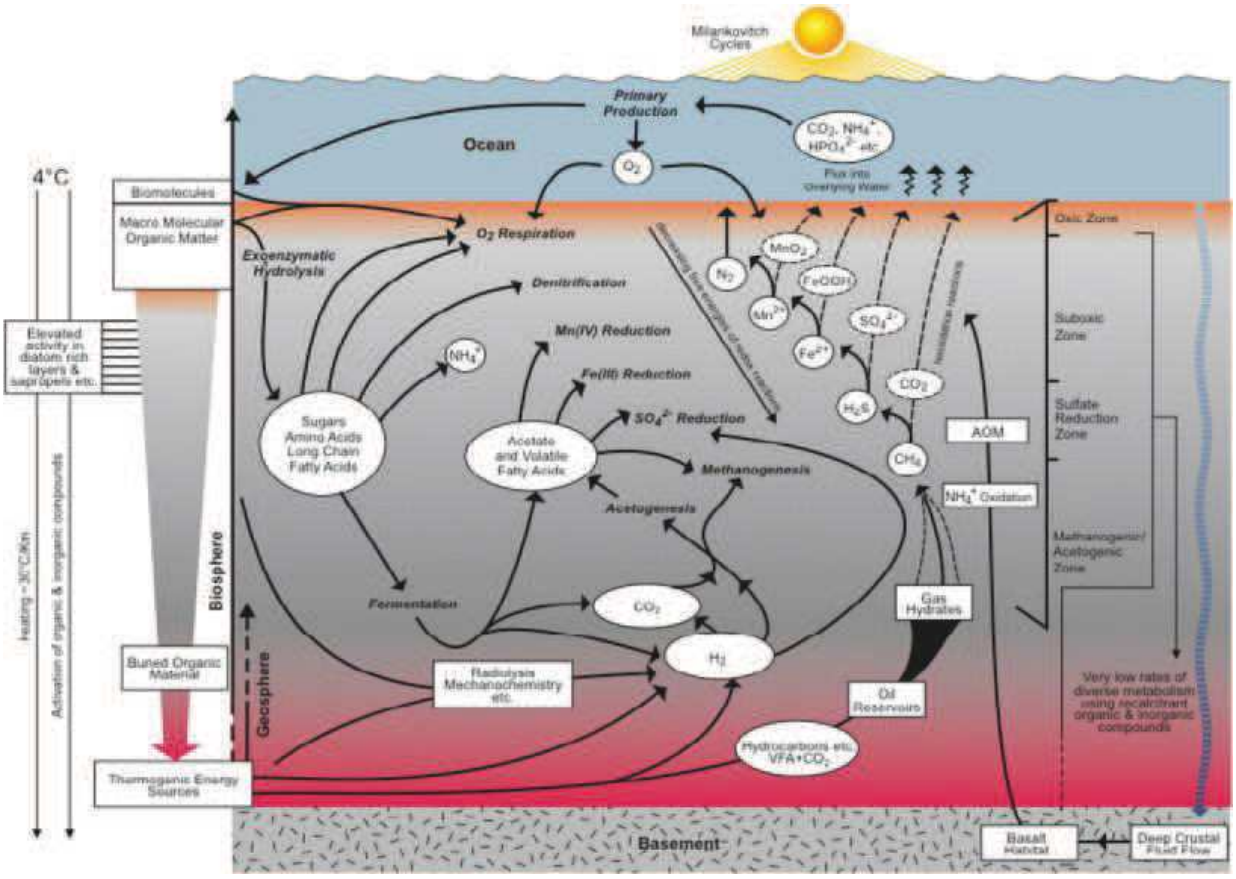


Figure 14 : Dégradation et transformation de la MO dans les sédiments marins. D'après Parkes *et al*. (2014)

1.2.3. La biosphère de subsurface

L'exploration de la biosphère profonde a été initiée dans les années 1970 dans le cadre de programmes internationaux et pluridisciplinaires de forages océaniques (DSDP : Deep Sea Drilling Project à partir de 1966, ODP : Ocean Drilling Program à partir de 1983, IODP : Integrated Ocean Drilling Program à partir de 2003 et depuis 2013, IODP : International Ocean Discovery Program). Il s'agit d'une collaboration internationale (26 nations) dédiée à la découverte des océans dans le but de décrypter et d'explorer l'histoire et la dynamique de la Terre à travers l'observation des fonds marins. L'étude des communautés microbiennes hébergées par les sédiments marins profonds a été facilitée par l'amélioration des performances des forages scientifiques au fil des années. Ces micro-organismes qui vivent dans les sédiments marins profonds appartiennent à ce qui est appelé la biosphère de subsurface et s'étend jusqu'à la croûte océanique basaltique. A travers des fractures et stylolithes (surfaces de dissolution), cet écosystème bien qu'hostile, fournit un habitat pour la vie microbienne (Teske *et al*., 2013). La biosphère de subsurface, définie selon des critères écologiques, est considérée comme étant l'horizon sédimentaire où les communautés microbiennes de la colonne d'eau sont remplacées par des communautés microbiennes typiques des sédiments alors que la biosphère superficielle est caractérisée par les sédiments situés entre l'interface eau–sédiment et la limite supérieure de la biosphère de subsurface (Teske et Sørensen, 2008).

1.2.3.1. Echantillonnage

1.2.3.1.1. Outils de forage profonds

Les outils de forage ont été adaptés de l'industrie pétrolière ou développés dans le cadre du programme international de forages océaniques profonds. Deux navires foreurs (le *Joides Resolution* et le *Chikyu*) et des plateformes de forage (Figure 15), appartenant au programme, sont à la disposition des scientifiques sous l'égide de trois organisations d'exécution, le ministère Japonnais de l'Education, de la Culture, des Sports, de la Science et de la Technologie (MEXT), la Fondation Nationale pour la Science des Etats-Unis (NSF) et le

consortium Européen ECORD (European Consortium for Ocean Research Drilling) qui servent d'opérateurs scientifiques. Les plateformes de forage, adaptées à des missions spécifiques, sont utilisées dans les zones couvertes de glaces ou les zones à faible profondeur (<100 m). Les navires foreurs, équipés de systèmes de forage et de laboratoires permanents, sont quand à eux utilisés en haute mer. La technologie de forage sans tube prolongateur dite «Riserless», à bord du *Joides Resolution,* utilise de l'eau de mer comme fluide de forage, pompée de manière continue à travers un tube de forage, afin de lubrifier et refroidir le trépan situé à l'extrémité du système. Le navire foreur *Chikyu* dont le nom signifie "planète Terre" en japonais est équipé de la dernière technologie de forage dite «Riser». Une colonne (ou tube prolongateur) placée autour du tube de forage permet l'évacuation des débris du forage vers le navire grâce à la circulation d'un courant de boues continu. Cette technique est essentielle pour les forages très profonds car elle protège le tube de forage et le trépan des débris de forage, des températures élevées et maintient la pression afin d'éviter un effondrement de la zone étudiée. Cette technique devrait permettre de forer jusqu'à 7 km sous la surface du plancher océanique.

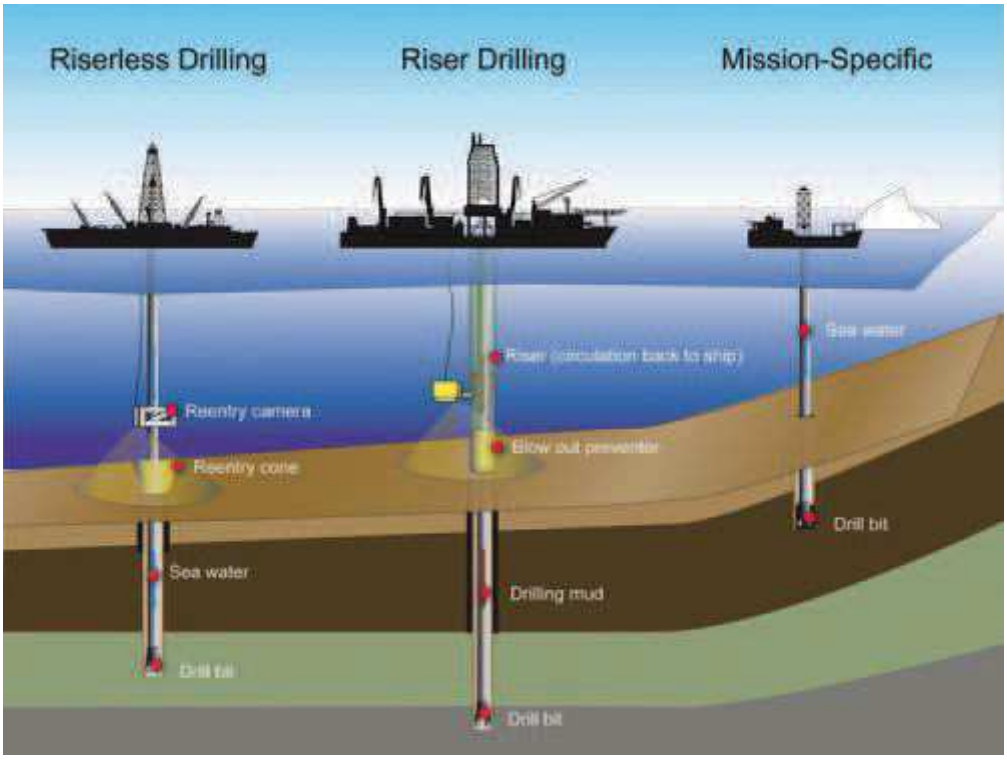


Figure 15 : Différentes plateformes de forages IODP.
(http://www.iodp.org/ships/platforms)

1.2.3.1.2. Contrôle de la contamination

Obtenir des échantillons représentatifs des milieux profonds est un des grands challenges en microbiologie de subsurface. En effet, l'introduction de contaminants lors des étapes de forage et d'échantillonnage doit être maîtrisée et contrôlée afin de garantir des échantillons exploitables. Une attention particulière est portée sur l'introduction potentielle de micro-organismes non-indigènes via le matériel et les fluides de forage au niveau de la carotte de sédiment. Le risque est d'autant plus élevé dans les environnements très profonds où les micro-organismes sont peu abondants. Deux techniques sont couramment utilisées lors des campagnes de forages profonds : les traceurs chimiques et les traceurs particulaires. Les traceurs chimiques sont basés sur l'hypothèse que les micro-organismes vont suivre le flux de fluides de forage (eau de mer de surface/boues) de la même façon que les traceurs. Un traceur idéal doit être chimiquement inerte, absent dans l'environnement *in situ* et facilement détectable à de très faibles concentrations (McKinley et Colwell, 1996). Habituellement, le traceur ajouté aux fluides de forage est le perfluorocarbone qui sera quantifié par chromatographie gazeuse sur échantillons collectés (Smith *et al*., 2000). Les microsphères fluorescentes, de taille similaire aux micro-organismes indigènes (0,5 à 1,0 µm) sont utilisées en tant que traceur particulaire. Une poche de 20 mL d'une suspension de microsphères (~10^{10} sphères/mL) est introduite dans le carottier et les microsphères relarguées au cours du carottage sont observées par microscopie à épifluorescence (Smith *et al*., 2000). Les deux types de tests menés sur le *Joides Resolution* suggèrent que l'intrusion du fluide de forage est la plupart du temps négligeable, de l'ordre de 1 à 10 micro-organismes/g de sédiment. Le niveau de contamination peut dépendre de la composition du sédiment. En effet, les basaltes fracturés sont plus sensibles aux contaminations par les eaux de forages. Les microsphères fluorescentes ne sont que très rarement détectées à l'intérieur de la carotte de sédiment qu'il soit meuble ou consolidé. Bien que les contaminations soient négligeables, un sous-échantillonnage de la carotte de sédiment est systématiquement effectué afin de prévenir d'éventuelles contaminations provenant de la périphérie de la carotte, la partie potentiellement la plus exposée aux fluides de forage. Toutes ces manipulations sont nécessaires afin d'évaluer les contaminations des échantillons destinés aux analyses microbiologiques et notamment ceux de faible biomasse pour ne pas altérer notre vision de la biosphère profonde.

1.2.3.2. Diversité procaryotique

1.2.3.2.1. Diversité moléculaire

Les communautés microbiennes des sédiments de subsurface présentent une large diversité de procaryotes ubiquistes et endémiques (Inagaki *et al*., 2003, 2006; Kormas *et al*., 2003; Newberry *et al*., 2004; Sørensen *et al*., 2004; Parkes *et al*., 2005, 2007; Biddle *et al*., 2006, 2008; Sørensen et Teske, 2006; Teske et Sørensen, 2008; Durbin et Teske, 2011; Breuker *et al*., 2013). Parmi les séquences bactériennes les plus abondantes, on retrouve les lignées de *Chloroflexi*, *Gammaproteobacteria*, *Planctomycetes* et la division candidate JS1, représentant plus de 60% de la diversité (Parkes *et al*., 2014). Les autres lignées bactériennes mises en évidence mais en plus faible abondance appartiennent aux *Alpha*−, *Beta*−, *Delta*− et *Epsilonproteobacteria, Bacteroidetes*, *Firmicutes, Actinobacteria, Spirochaetes* et les groupes OP8, OP11, NT-B2. Les séquences d'*Archaea* identifiées au sein des sédiments de subsurface comprennent une large fraction de lignées d'incultivés, dont beaucoup sont endémiques des sédiments de subsurface. Parmi les *Crenarchaeota*, on trouve les divisions candidates MCG (Miscellaneous Crenarchaeotic Group) et MBG−B (Marine Benthic Group B) fortement représentées mais aussi les MBG−A (Marine Benthic Group A) et le MHVG (Marine Hydrothermal Vent Group) en plus faible abondance. Les *Euryarchaeota*, généralement moins abondants que les *Crenarchaeota,* sont représentés par les MBG−D (Marine Benthic Group D) et les SAGMEG (South African Gold Mine groups 1&2). Les méthanogènes (*Methanosarcinales, Methanomicrobiales* et *Methanobacteriales*), les ANME (ANaerobic MEthanotrophic *Archaea*) et les archées hyperthermophiles (*Thermococcales, Methanococcales* et *Archaeoglobales*) sont quant à elles moins abondantes. Le phylum des *Thaumarchaeota* nouvellement décrit (Brochier-Armanet *et al*., 2008) est représenté par les MG−I (Marine Group I) (Figure 16).

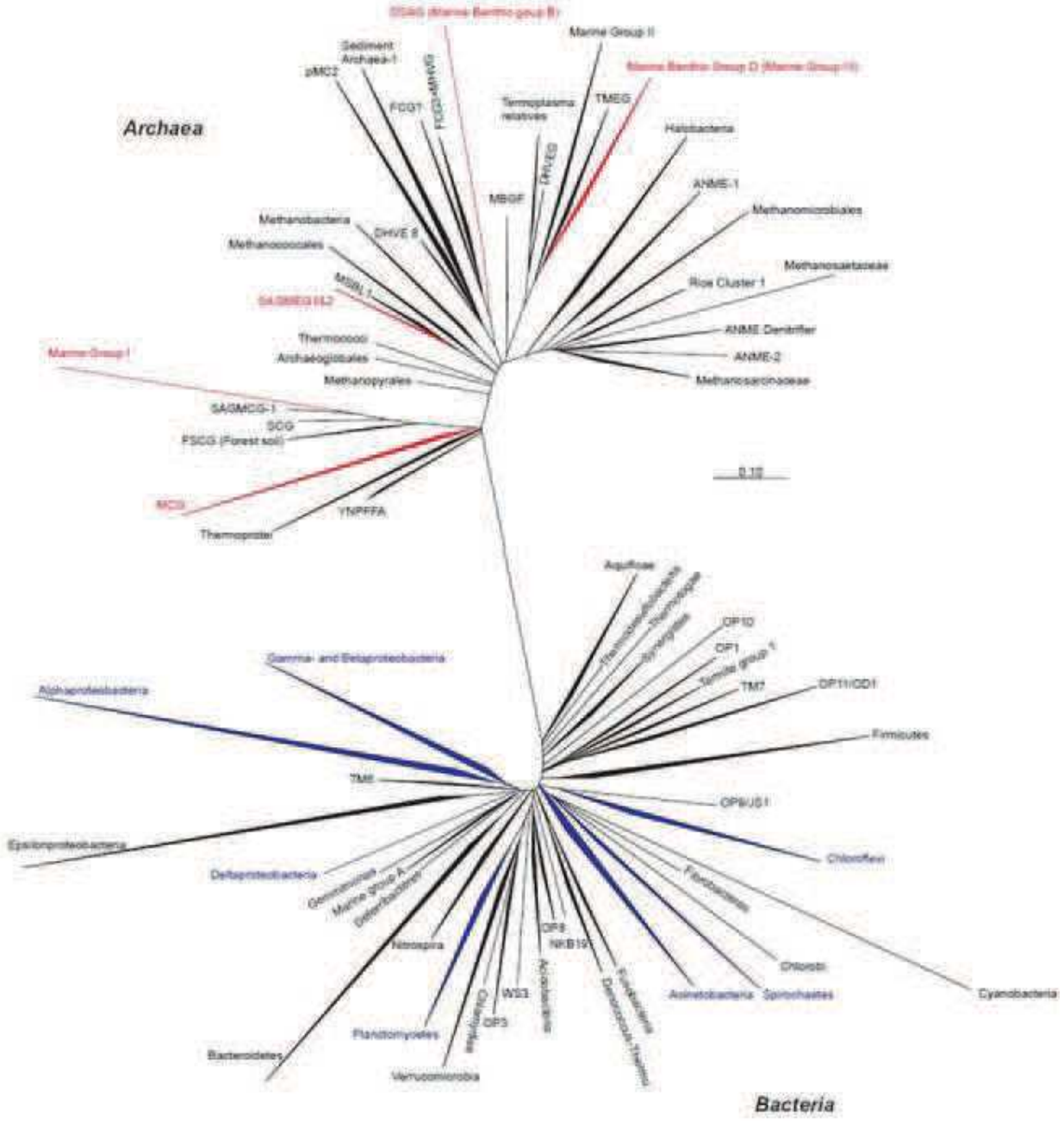


Figure 16 : Arbre phylogénétique des séquences procaryotiques du gène de l'ARNr 16S. D'après D'Hondt *et al.*, (2007)

Les branches rouges et bleues représentent respectivement les lignées archéennes et bactériennes fréquemment détectées dans les sédiments de subsurface.

1.2.3.2.2. Diversité cultivable

Les représentants cultivables procaryotes issus de sédiments de subsurface appartiennent principalement pour les bactéries aux *Proteobacteria, Firmicutes, Actinobacteria* et *Bacteroidetes* et pour les archées aux *Euryarchaeota* méthanogènes (Bale *et al*., 1997; Barnes *et al*., 1998; Inagaki *et al*., 2003; Mikucki *et al*., 2003; D'Hondt *et al*., 2004; Sub *et al*., 2004; Toffin *et al*., 2004, 2005; Lee *et al*., 2005; Takai *et al*., 2005; Kendall *et al*., 2006; Teske, 2006; Batzke *et al*., 2007; Kobayashi *et al*., 2008; Parkes *et al*., 2009; Imachi *et al.,* 2011). Certains de ces isolats montrent des capacités métaboliques bien spécifiques des conditions *in situ* comme la bactérie sulfato− réductrice piézophile *Desulfovibrio profundus* (Bale *et al*., 1997), ou bien les archées méthanogènes des genres *Methanoculleus* (Mikucki *et al*., 2003), *Methanococcus* (Kendall *et al*., 2006), *Methanosarcina, Methanobacterium, Methanococcoides* et *Methanobrevibacter* (Imachi *et al*., 2011). A ce jour, la plupart des lignées procaryotes détectées par les techniques moléculaires restent incultivées. On estime en effet à 0,25% la diversité microbienne des sédiments marins profonds cultivée (Amann *et al.,* 1995, Rappé et Giovannoni, 2003). Ceci s'explique principalement par la difficulté à reproduire en laboratoire l'ensemble des contraintes physico-chimiques propres à cet écosystème. Bien que les nouvelles approches moléculaires, comme la métagénomique, l'analyse de génome *single-cell* ou encore la métatranscriptomique, aient permis d'améliorer considérablement les connaissances sur les capacités génétiques des micro-organismes incultivés, les caractéristiques écophysiologiques (température, pression, gamme de substrat favorables à la croissance) ne peuvent être déterminées que par des approches culturales (Parkes *et al*., 2014).

1.2.3.2.3. Abondance et activité

Bien que les récents comptages cellulaires montrent que la biosphère profonde a été surestimée de près de 50% en 1998 par Whitman et son équipe (Whitman *et al*. 1998), elle constitue tout de même une large fraction de la biomasse terrestre. Réalisés sur des échantillons prélevés de régions particulièrement oligotrophes et de zones de forte productivité, à différents endroits du globe, de récents comptages cellulaires ont montré que la

biosphère profonde représenterait entre 0,18 et 3,6% de la biomasse procaryotique sur Terre avec 2.9×10^{29} cellules (Kallmeyer *et al*., 2012).

L'estimation de la biomasse procaryotique dans les sédiments marins la plus récente, incluant les derniers échantillonnages jusqu'à 1922 mètres sous la surface du plancher océanique a été revue à 5.39×10^{29} cellules (Parkes *et al*., 2014). Plus élevée que précédemment annoncé, cette étude intègre des sédiments riches en matière organique, tels les dépôts d'hydrates de gaz et les réservoirs de pétrole. Que la zone soit oligotrophe ou au contraire bien plus riche en matière organique, la distribution du nombre de cellules est toujours la même et diminue de façon logarithmique avec la profondeur (Figure 17).

Les comptages cellulaires, utilisés pour estimer l'abondance de la biosphère profonde, ne permettent pas de discriminer les cellules vivantes, actives, mortes ou en dormance. Pour étudier les communautés actives, cibler des molécules rapidement dégradées, semble être la meilleure approche. C'est par des techniques basées sur les ARNr et sur les lipides membranaires intacts (IPL), que l'abondance et l'activité des communautés procaryotes de subsurface ont été démontrées (Zink *et al*., 2003; Mauclaire *et al*., 2004; Schippers *et al*., 2005; Biddle *et al*., 2006; Schippers et Neretin 2006; Lipp *et al*., 2008, Orsi *et al*., 2013b). Cependant, la dominance des bactéries ou des archées n'est pas encore définie car les résultats sont variables selon les techniques employées, pour une même zone géographique. En effet, au niveau de la marge du Pérou, des analyses de CARD-FISH combinées à de la Q-PCR et de métatranscriptomique, ont montré que les bactéries dominaient les communautés procaryotes (Schippers *et al*., 2005, Orsi *et al*., 2013b) tandis qu'une autre étude couplant FISH, analyse des IPL et extraction des ARNr suggèrent que la partie active des communautés microbiennes serait dominée par les archées (Biddle *et al*., 2006). Cependant, bien que les IPL soient considérés comme des biomarqueurs de la biomasse active, de récentes études montrent que les IPL issus de bactéries seraient dégradés plus rapidement que ceux issus d'archées (Schouten *et al*., 2010; Logemann *et al*, 2011; Xie *et al*., 2013). Par conséquent, les précédentes études, basées sur les IPL, auraient probablement surestimé la biomasse microbienne et la dominance observée des archées n'exclut pas pour autant la possibilité d'une biosphère profonde dominée par les bactéries.

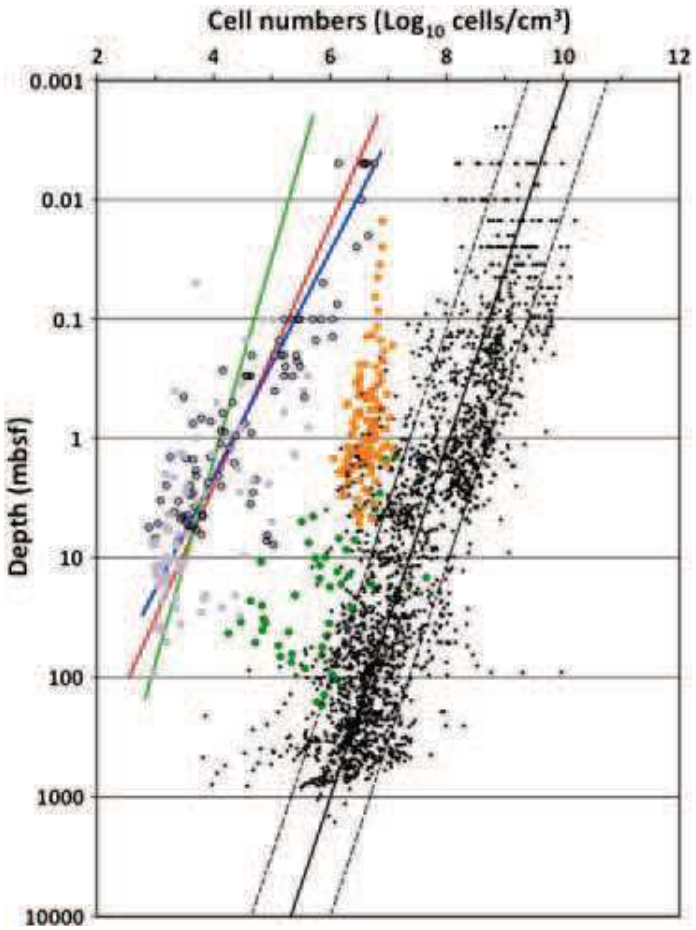


Figure 17 : Distribution de cellules procaryotes dans les sédiments marins. D'après Parkes *et al*. (2014)

Les cercles noirs représentent les comptages cellulaires effectués initialement par Parkes *et al*., 1994 au niveau de l'océan Pacifique auxquels de nouveaux comptages, effectués au niveau de l'océan Atlantique et de la mer Méditerranée, ont été ajouté. Les cercles oranges représentent les comptages cellulaires effectués au niveau de volcans de boue au niveau de la mer Noire. Les cercles verts représentent les comptages cellulaires effectués à partir d'échantillons hydrothermaux. Les cercles mauves représentent les comptages cellulaires effectués par Kallmeyer *et al*., 2012 au niveau du Gyre subtropical du Pacifique Sud.

1.2.3.3. Diversité virale

Les études portant sur les virus ont été effectuées au niveau des couches sédimentaires supérieures et leur abondance est généralement comprise entre 10^4 et 10^9 virus par cm^3 (Engelhardt *et al*., 2014). Bien que l'abondance des particules virales diminue avec la

profondeur, elle reste habituellement supérieure à celle des cellules microbiennes (Middelboe *et al.,* 2006, 2011; Danovaro *et al*, 2008; Siem-Jørgensen *et al*., 2008) et les infections virales seraient responsables de plus de 80% de la mortalité microbienne. La lysogénie semble être le mode de reproduction virale majoritaire dans les sédiments marins profonds, c'est-à-dire qu'après l'infection, le génome viral se réplique comme un prophage avec celui de l'hôte. En effet, près de la moitié des isolats bactériens de subsurface héberge des prophages (Engelhardt *et al*., 2011). L'omniprésence de la lysogénie pourrait avoir des implications intéressantes pour la survie cellulaire dans cet écosystème où les sources d'énergie sont limitées et où les micro-organismes sont soumis à une forte pression de sélection (Anderson *et al*., 2013). Certains phages lysogènes peuvent induire la répression des gènes du métabolisme de l'hôte et ainsi limiter les processus métaboliques de l'hôte lorsque les conditions ne sont pas favorables (Paul, 2008). Cette caractéristique des phages lysogènes pourrait être particulièrement utile dans les environnements profonds où l'accessibilité aux sources d'énergie est limitée. Si l'impact des virus dans le recyclage des nutriments et la production de matière organique labile est démontré dans les sédiments superficiels (Danovaro *et al*., 2008), il n'existe pas de données comparables disponibles au niveau de la biosphère profonde. La compréhension de la distribution virale et de son rôle écologique dans les sédiments marins profonds n'en est qu'à ses débuts et soulève de nombreuses questions quant à l'origine des virus, leur devenir et leur impact sur les communautés microbiennes dans cet environnement (Middelboe *et al*., 2011)

1.2.3.4. Diversité eucaryote

Si les études portant sur les bactéries et les archées sont nombreuses, celles portant sur les communautés de micro-eucaryotes restent largement minoritaires alors qu'elles pourraient avoir un rôle tout aussi important en contrôlant l'abondance et la composition des communautés bactériennes et archéennes. De cette façon, les micro-eucaryotes sont susceptibles de jouer un rôle clé dans les grands cycles biogéochimiques des sédiments marins profonds.

1.2.3.4.1. Micro-eucaryotes

Les études moléculaires et culturales s'intéressant spécifiquement aux micro-eucaryotes ont prouvé leur présence dans les écosystèmes marins profonds notamment au niveau de sources hydrothermales (Edgcomb *et al*., 2002; López-García *et al*., 2003, 2007; Burgaud *et al.,* 2009, 2010; Le Calvez *et al*., 2009), d'environnements anoxiques (Stoeck et Epstein 2003; Stoeck *et al.,* 2003, 2006, 2010; Takishita *et al.,* 2005), de bassins anoxiques hypersalés (Alexander *et al*., 2009; Stock *et al*., 2012), de zones de suintements froids riche en méthane (Takishita *et al*., 2007; Nagahama *et al*., 2011) mais aussi au niveau de sédiments marins superficiels et profonds (Biddle *et al*., 2008; Scheckenbach *et al*., 2010; Edgcomb *et al*., 2011; Pawlowski *et al*., 2011; Orsi *et al.,* 2013a,, 2013b; Xu *et al*., 2014). Les séquences d'ARNr 18S mises en évidence dans les sédiments marins englobent la majorité des lignées déjà décrites dans le domaine eucaryote, parmi les *Fungi*, *Alveolata, Euglenozoa, Stramenopiles, Rhizaria* et *Cercozoa* pour les OTUs les plus abondantes. La composition des communautés eucaryotes de subsurface n'est pas uniforme en fonction de la profondeur et des différents sites d'échantillonnage, ce qui indique que les conditions biogéochimiques influencent largement la composition des communautés. De plus, une plus large diversité eucaryote est observée dans les premières couches sédimentaires, ce qui suggère que ces communautés eucaryotes auraient pour origine la colonne d'eau (Edgcomb *et al.,* 2011).

1.2.3.4.2. Champignons

Les premières analyses de diversité eucaryote à partir des sédiments marins profonds sont récentes (Edgcomb *et al*., 2011; Orsi *et al*., 2013a). C'est au niveau de la marge et de la fosse du Pérou que les micro-eucaryotes ont été mis en évidence jusqu'à 35 puis 48 mètres sous la surface du plancher océanique. La plus grande diversité taxonomique a été observée dans les échantillons les moins profonds ce qui n'est pas surprenant puisque à cette profondeur (1,75 m) toute une variété de substrats organiques est susceptible d'être disponible en raison de la sédimentation et des activités des organismes benthiques. Parmi les séquences détectées, ce sont celles des champignons qui dominent dans les banques de clones construites à partir des ADN et ARN extraits. Dominées par les *Basidiomycota,* les populations fongiques, sont

donc réellement actives au sein de la biosphère profonde. Bien que les séquences détectées soient de proches parents de champignons terrestres, ces populations de champignons de subsurface présentent nécessairement des adaptations aux conditions rencontrées *in situ*. La frontière entre les champignons terrestres et marins n'est actuellement pas claire. Historiquement, les champignons marins obligatoires ont été décrits comme étant exclusivement capables de croître et de se reproduire dans un habitat marin ou estuarien à l'inverse des champignons marins facultatifs, capables de croître et de se reproduire quelque soit l'habitat, marin ou terrestre (Kohlmeyer et Kohlmeyer, 1979). Alors que les champignons sont de plus en plus retrouvés dans les environnements marins, et notamment des champignons ubiquistes, cette différenciation claire entre les champignons marins et terrestres ne semble plus si évidente. Des interrogations demeurent aujourd'hui sur les communautés fongiques actives de subsurfaces : regroupent-elles des organismes terrestres en dormance, des organismes à forte capacité d'adaptation ou bien sont-elles vraiment spécifiques du milieu marin ? Pour répondre à ces questions il est nécessaire d'avoir un aperçu de leur diversité taxonomique et de leur niveau d'activité métabolique au sein de la biosphère profonde. Une récente étude va dans ce sens en ciblant spécifiquement les ARNm (Orsi *et al.,* 2013b). Ces premières données sur l'expression génétique microbienne de subsurface confirment les études précédentes et malgré leur faible activité métabolique, les champignons sont actifs au sein de la biosphère profonde. Révélés jusqu'à 159 mètres sous la surface du plancher océanique de la marge du Pérou, les champignons représentent 20% des transcrits messagers à 5 mbsf et 5% à 159 mbsf. Capables de dégrader toute une variété de substrats organiques comme les glucides, lipides et protéines, les champignons devraient avoir un rôle dans le recyclage du carbone organique dans les sédiments marins profonds. L'analyse des transcrits fongiques dans différentes zones géographique permettra à terme d'obtenir une vision plus claire de l'implication des communautés fongiques dans la part active de la biosphère profonde.

Le but de cette partie était de présenter l'écosystème sédimentaire marin et de montrer que malgré sa complexité, une diversité de micro-organismes procaryotes et eucaryotes peut persister à plusieurs centaines de mètres sous le plancher océanique. Dans la partie suivante, nous verrons que l'augmentation constante des données moléculaires a permis de révéler une

diversité fongique marine bien supérieure à ce que l'on pensait auparavant. Dans le chapitre qui suit, publié en 2014 dans l'ouvrage intitulé « The ecological genomics of fungi » (Mahé et al., 2014), je me suis impliquée dans la présentation des techniques de culture et d'isolement utilisées pour étudier les champignons marins ainsi que l'état de l'art des connaissances de la diversité fongique des sédiments marins.

1.3. Les champignons marins

15 Fungi in Deep-Sea Environments and Metagenomics

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Fungi in Oceans: An Overview

Oceans harbor a broad diversity of photosynthesis- and chemosynthesis-based ecosystems, from coastal waters to the deep biosphere that is, oceanic waters below 1000 meters (Jannasch & Taylor, 1984), and a wide diversity of micro-organisms involved in all biogeochemical cycles (Fig. 15.1). Fungi in oceans form an ecologically defined group of filamentous ascomycetes, their anamorphs, and yeasts (Kohlmeyer & Kohlmeyer, 1979). Although ecologically important relationships with other organisms (e.g., pathogens and symbionts of algae, higher plants and animals, as well as an important role as decomposers) have been clearly demonstrated, few species of marine fungi have been listed to date. Recent reports of fungi in the deep oceans have provided insights into their global diversity and ecological role. The constant increase of molecular data using clone libraries or high-throughput sequencing is permitting a revision of the definition of marine fungi.

History and Definitions: The Concept of Marine Fungi

From the first comprehensive study on marine fungi by Barghoorn and Linder (1944) to the first exhaustive and comprehensive book dealing with marine mycology (Kohlmeyer & Kohlmeyer, 1979), and from the initial recovery of fungal communities at deep-sea extreme environments (Nagahama, Hamamoto, et al., 2001) to one recent exhaustive review dealing with deep fungal communities (Nagano & Nagahama, 2012), marine fungi have

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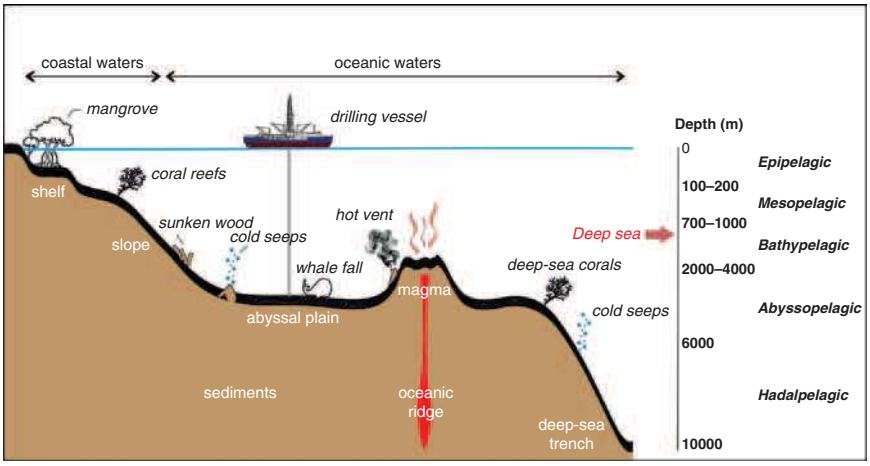

Figure 15.1 Schematic transversal section of the earth highlighting different marine ecosystems (not to scale).

usually been considered as exotic microorganisms that only fascinate a limited panel of scientists. The first definition of a marine fungus was produced by Johnson and Sparrow (1961) and based on an ability to grow at seawater concentrations. This physiologically based postulate was later criticized by Kohlmeyer and Kohlmeyer (1979) who suggested a broad ecological definition dividing marine fungi into obligate and facultative groups. This strict dichotomy between obligate and facultative marine fungi led to a distinction between fungi "that grow and sporulate exclusively in a marine or estuarine habitat from those from freshwater or terrestrial milieus able to grow and possibly to sporulate in the marine environment" (Kohlmeyer & Kohlmeyer, 1979: 42). Currently, the interest in marine fungi has clearly exploded, and this is reflected in several recent reviews (Shearer, Descals, et al., 2007; Jones, Sakayaroj, et al., 2009; Jones, 2011; Nagahama & Nagano, 2012; Nagano & Nagahama, 2012; Richards, Jones, et al., 2012). The idea here is not to provide yet another updated review dealing with marine mycology but rather to discuss the actual concept of marine fungi and to attempt to update the preceding definition.

The definition proposed by Kohlmeyer and Kohlmeyer (1979) has been widely accepted by the scientific community but, as is usual, has not escaped debate. The dilemma is always whether or not to consider facultative marine fungi because the split generated by this definition has led to a kind of segregation against facultative marine fungi. This can be easily explained from the analytical strategy used by scientists: (a) historically, direct observations of in situ fungal structures have allowed the identification and characterization of

numerous obligate marine fungi and (b) untargeted culture-based approaches have mostly revealed strains commonly isolated in terrestrial habitats and thus defined as facultative marine fungi. The fact that facultative marine fungi were receiving little attention led some scientists to propose an initial exegesis, stating that "terrestrial species turn out far too regularly from macrophytic detritus to be dismissed lightly" (Raghukumar & Raghukumar, 1999: 26). In a form of response, Kohlmeyer and Volkmann-Kohlmeyer (2003) commented on the plate method, which often reveals common dust and wind-born forms and concluded that advocates of the untargeted culture-based method must be responsible for clarifying whether such facultative marine fungi occur as dormant spores or not.

Some studies have proven facultative marine fungi with terrestrial representatives to have an ecological role in marine environments, for example, *Aspergillus sydowii* pathogen of corals (Geiser, Taylor, et al., 1998) or *Fusarium oxysporum* pathogen of crustaceans (Khoa & Hatai, 2005). Metabolic profile analyses of facultative marine fungi have also helped to clarify their ecological role because marine-derived fungi synthesize a broad spectrum of secondary metabolites. Some have been retrieved from both terrestrial and marine strains (e.g., wentilactone from *Aspergillus wentii*). But in general, many compounds harvested from marine-derived fungi differ from those of their terrestrial representatives (Bhakuni & Rawat, 2005), for example, nitrogenous compounds such as heptapeptides from *Acremonium persicinum* showing cytotoxicity against brine shrimps and toward tumor cell lines (Chen, Song, et al., 2012), polyketides from *Phoma herbarum* showing antiviral activity (Zhang, Han, et al., 2012), and terpenoids from *Penicillium chrysogenum* showing antibacterial activity (Gao, Li, et al., 2011). This hidden wealth of resources has not only opened up a new era in drug research but also suggests that secondary metabolites from marine-derived fungi may act as a chemical defense mechanism in oceans and that marine-derived fungi are not only dormant spores in this ecosystem (Damare, Singh, et al., 2012). Although the synthesis of exotic secondary bioactive molecules may indicate a stress response, it definitely indicates metabolic activity. Going round in circles, Capon, Ratnayake, et al. (2005) revealed that the terrestrial fungal strain *Aspergillus unilateralis* was able to synthesize Trichodermamide B dipeptide, a typical marine-derived metabolite, when cultured in media enriched with sodium chloride (NaCl). This result suggests that metabolic expression profiles are influenced by environmental rather than genetic factors.

What if this old debate regarding obligate and facultative marine fungi can be laid to rest just by accepting that it is a non-issue? Scaling up the methods used to define marine fungi might provide a kind of consensus. Most marine mycologists have discussed Kohlmeyer and Kohlmeyer's definition summarizing marine fungi as an ecological rather than taxonomic group (Hyde, Jones, et al., 1998; Shearer, Descals, et al., 2007; Gao, Li, et al., 2008;

Burgaud, Le Calvez, et al., 2009; Jones, Sakayaroj, et al., 2009; Das, Lyla, et al., 2009; Nagahama & Nagano, 2012). However, based on this definition, marine fungi are truly initially defined by culture efficiency or direct observations rather than by ecological traits. Culture-based and in situ observation approaches are biased by unculturable fungi, endophytes, cryptic species, strict parasites, fast growing strains, dormant spores germination and indeed are strongly selective because they only allow detection of a tiny fraction of fungi in environmental samples. The huge advances in marine molecular ecology have led to a reversal of these common approaches and thus constrain to update Kohlmeyer and Kohlmeyer's definition through the use of informative keywords. The keywords mostly used to describe fungal communities in marine environments are *isolation*, *direct detection*, *diversity*, *adaptation*, and *ecological role*. *Activity* appears as another keyword that fits well to an updated version of the Kohlmeyer and Kohlmeyer's definition. Fungal communities in oceans can thus be divided into three levels of occurrence: (a) strict endemic active marine fungi, (b) ubiquist metabolically active marine fungi, and (c) ubiquist passive fungi. By combining this three-level classification with Kohlmeyer and Kohlmeyer's definition, a pattern of fungal diversity, based on a functional scale, can be drawn up. The current new era of metagenomics and metatranscriptomics is providing enough power to fit this pattern.

Diversity and Ecological Roles of Fungi in Oceans

Marine as compared with terrestrial ecosystems have been little studied by mycologists. Currently, 549 obligate marine species of higher fungi (i.e., *Ascomycota* and *Basidiomycota*) have been described based on the Kohlmeyer and Kohlmeyer's definition, with only 54 species since the year 2000 (Jones, 2011). Only a few basal lineage species have been identified from marine environments (Gleason, Küpper, et al., 2011). In littoral and sublittoral regions, fungal species are mainly lignicolous (Jones, 2011) and algicolous (Bugni & Ireland, 2004), but have also been isolated from substrates such as marine plants, for example, partly submerged plants such as *Spartina* or permanently submerged plants such as *Posidonia*, sand, corals, calcareous algae, mollusk shells, hydrozoan exoskeletons, or annelid tubes (Jones, 2011). Microscopic fruiting bodies were directly examined on marine substrates or isolated on culture media using ascospores from ascoma as inoculum. Cultivation and molecular approaches were proved more appropriate in open-sea ecosystems. Indeed, the first deep-sea studies were based on the observation of fruiting bodies on wood- or polyurethane-covered panels (Jones, 2011), which made it difficult to differentiate between indigenous fungi and contaminants (Kohlmeyer & Kohlmeyer, 1979). Although descriptions based on an examination of fruiting bodies on marine substrates have

been widely used, a culture-based approach has led to the description of many marine fungi in various marine niches at different depths (Table 15.1).

When molecular methods were used, with or without a fungal-specific approach (Table 15.2), the sequences of higher fungi retrieved were often closely affiliated to terrestrial fungi (Richards, Jones, et al., 2012). Yeasts appeared dominant because many genera were retrieved, for example, *Cryptoccocus*, *Rhodotorula*, *Candida*, and *Debaryomyces* (Bass, Howe, et al., 2007; Le Calvez, Burgaud, et al., 2009). Environmental sequences close to the yeast *Malassezia* were frequently found in eukaryotic- or fungal-specific studies in different marine extreme environments and have been clustered in a group formally known as "hydrothermal and/or anoxic marine yeasts" (Dawson & Pace, 2002; López-García, Vereshchaka, et al., 2007; Bass, Howe, et al., 2007; Le Calvez, Burgaud, et al., 2009; Jebaraj, Raghukumar, et al., 2010; Edgcomb, Beaudoin, et al., 2011). Significant numbers of *Malassezia* sequences were retrieved in hypersaline anoxic basins in the Mediterranean Sea (Alexander, Stock, et al., 2009; Edgcomb, Orsi, et al., 2009). Filamentous forms, such as *Aspergillus*, *Exophiala*, and *Tilletiopsis* are fairly well represented in cultures (Damare, Raghukumar, et al., 2006; Burgaud, Le Calvez, et al., 2009) and their presence has been confirmed using molecular methods (López-García, Rodríguez-Valera, et al., 2001; Stoeck, Hayward, et al., 2006; Bass, Howe, et al., 2007). Sequences close to terrestrial *Agaricomycetes* were also reported in deep marine ecosystems, for example, *Exidia*, *Coprinopsis*, or *Antrodia* (Bass, Howe, et al., 2007; Alexander, Stock, et al., 2009; Le Calvez, Burgaud, et al., 2009; Jebaraj, Raghukumar, et al., 2010) leading to debate about the activity of terrestrial fungi in such extreme environments and the need to investigate functional diversity rather than genetic diversity. Basal fungal lineages were detected in various marine systems by using molecular methods (i.e., *Blastocladiomycota, Chytridiomycota,* and *Cryptomycota*) but most of them have not yet been affiliated to known isolated representatives (Richards, Jones, et al., 2012).

Another level of fungal occurrence was revealed by pyrosequencing eukaryotic V4 and V9 tags from marine anoxic waters. Potential sources of error that might inflate the apparent level of diversity were discussed (nucleotide misincorporation, read errors, chimaera formation) but fungal diversity was much higher than expected with 1.5 to 4 percent of unique fungal tags depending on whether the markers, V4 or V9 marker was used (Stoeck, Bass, et al., 2010). Sequencing of the eukaryotic V9 diversity tags from sediment samples (686–6,326 m) using Roche 454-pyrosequencing revealed 101 operational taxonomic units (OTUs) in six samples, as compared to the total eukaryotic richness of 8309 OTUs representing 1.2 percent of the total eukaryotic diversity (Pawlowski, Christen, et al., 2011). A vertical pattern became apparent because most of the OTUs were obtained at lower depths. Fungi do not constitute a major component of the total eukaryotic OTUs recovered

Table 15.1 Fungal phyla retrieved by cultivation methods from different marine samples at various depths.

Reference	Location and Depth	Kind of Sample Processed	Phylogenetic Affinities
Nagahama, Hamamoto, et al., 2001	Sagami and Suruga Bay, Japan (1,000 to 11,000 m)	Superficial sediments Animals	*Ascomycota* *Basidiomycota*
Biddle, House, et al., 2005	Peru Margin (150 m)	Sediments (30 to 157 m below subfloor)	*Ascomycota*
Raghukumar, Raghukumar, et al., 2004	Chagos Trench Indian Ocean (5,904 m)	Sediments (0 to 370 cm below subfloor)	*Ascomycota*
Damare, Raghukumar, et al., 2006	Central Indian Basin (4,900 to 5,390 m)	Superficial sediments	*Ascomycota*
Singh, Raghukumar, et al., 2010	Central Indian Basin (4,000 to 5,700 m)	Superficial sediments	*Ascomycota* *Basidiomycota*
Gadanho & Sampaio, 2005	Hydrothermal sites, MAR (800 to 3,150 m)	Water: 3 to 5 m above the sea floor	*Ascomycota* *Basidiomycota*
Burgaud, LeCalvez, et al., 2009	Hydrothermal sites, MAR and EPR (700 to 3,650 m)	Water, sediments, mineral samples, animals	*Ascomycota* *Basidiomycota*
Le Calvez, Burgaud, et al., 2009	Hydrothermal sites, MAR (860 & 1,700 m) and EPR (2,630 m)	Animals, mineral samples	*Ascomycota*
Burgaud, Arzur, et al., 2010	Hydrothermal sites, MAR and Lau Basin: Pacifique (900 to 3,630 m)	Water, animals, experimental microcolonizer, shrimp sloughs	*Ascomycota* *Basidiomycota*
Jebaraj & Raghukumar, 2009	Arabian Sea (14 & 26 m)	Anoxic superficial sediments	*Ascomycota*
Jebaraj, Raghukumar, et al., 2010	Arabian Sea (3, 25 & 200 m)	Anoxic superficial sediments, water	*Ascomycota* *Basidiomycota*
Mouton, Potsma, et al., 2012	St Helena Bay (South Africa) (8, 15, 28, 32 61 m)	Superficial sediments	*Ascomycota* *Basidiomycota*

EPR, East Pacific Rise, MAR, Mid-Atlantic Ridge.

Table 15.2 Fungal phyla recovered by fungal-specific molecular methods from different marine samples at various depths.

Reference	Location and Depth	Kind of Sample Processed	Phylogenetic Affinities
Bass, Howe, et al., 2007	Wreck of *Bismarck* (3,000 & 4,000 m)	Water	*Ascomycota*
	Wreck of *Titanic* (3,000 & 3,700 m)	Water	*Basidiomycota*
	Hydrothermal site, MAR (2,264 m)	Sediments, experimental microcolonizers	*Chytridiomycota*
	Drake Passage (250, 500, 2,000 & 3,000 m)	Water	
	Gulf of California (1,575 m)	Anoxic bacterial mat	
Lai, Cao, et al., 2007	South China Sea (350, 884, 1,123, 2,965 & 3,011 m)	Methane hydrate-bearing sediments	*Ascomycota* *Basidiomycota*
Le Calvez, Burgaud, et al., 2009	Hydrothermal sites, MAR and EPR (860 to 2,630 m)	Animals, mineral samples	*Basidiomycota* *Chytridiomycota*
Nagano, Nagahama, et al., 2010	Izu-Ogasawara Trench (7,111 & 9,760 m)	Sediments (0 to 110 cm below seafloor)	*Ascomycota* *Basidiomycota*
	Mariana Trench (10,131 m)	Sediments	*Cryptomycota*
	Sagami Bay, methane cold-seep (1,174 m)	Bacterial mats	
Nagahama, Takahashi, et al., 2011	Sagami Bay, methane cold-seep (830 to 1,200 m)	Sediments	*Ascomycota* *Basidiomycota* *Blastocladiomycota* *Cryptomycota*
Thaler, Van Dover, et al., 2012	Gulf of Mexico, methane cold-seep (2,400 m)	Sediments (0 to 30 cm below seafloor)	*Ascomycota* *Basidiomycota* *Chytridiomycota*

EPR East Pacific Rise, MAR Mid-Atlantic Ridge.

from marine environments with only 1 percent of fungal OTUs obtained from coastal seawaters (Monchy, Grattepanche, et al., 2012). However, new high-throughput technologies are definitely enhancing the assessment of the marine fungal diversity.

The functions of fungi in oceans and their impact have been poorly investigated because the attention has only been focused on describing the diversity. However, major terrestrial fungal functions have already been observed in ocean systems. Fungi play pivotal roles as lignocellulolytic decomposers of floating or sunken woody substrates in marine ecosystems such as mangroves or other coastal areas (Hyde, Jones, et al., 1998). Fungal decomposers split complex polymers into particulate organic matter that then becomes available to other organisms. Planktonic marine fungi (i.e., ascomycetous and basidiomycetous mycoplankton) were found associated with the decomposition of organic matter, nutrient, and carbon cycling (Gao, Johnson, et al., 2010) and displayed distinct lateral and vertical patterns. Mycoplankton diversity and composition were relatively well correlated to phytoplankton biomass and primary production in Hawaii coastal waters from 5 to 200 m depth. Gutiérrez, Pantoja, et al. (2011) indicated a third dynamic temporal pattern of mycoplankton distribution represented by the Humboldt Current System, a cold, low-salinity and nutrient-rich ocean current rising to the surface. A large fungal biomass was retrieved during productive periods and was associated with a bloom of extracellular enzymes hydrolyzing up to 90 percent of the organic molecules from marine photosynthetic producers during the seasonal peak of production. Mycoplankton, such as prokaryotes, appeared as important actors in marine processes because of their abundance, diversity, and active participation to the carbon cycle through the release of dissolved organic molecules. Fungi also intervene in other biogeochemical patterns, such as the N cycle occurring in marine sediments. Fungal strains isolated from anoxic or suboxic sediments were able to participate in anaerobic denitrification processes, reducing nitrate or nitrite under anaerobic conditions (Jebaraj & Raghukumar, 2009; Mouton, Postma, et al., 2012).

As in terrestrial ecosystems, marine fungi can be found associated with macro and microorganisms. Some marine fungal species (e.g., *Leiophloea*, *Pharcidia*, or *Mycosphaerella* genera) contract symbioses with microscopic algae or cyanobacteria to form lichens. Some are associated with macroalgae to form mycophycobioses, such as members of the *Blodgettia* and *Turgidosculum* genera (Kohlmeyer & Kohlmeyer, 1979; Hyde, Jones, et al., 1998). Sponges host an astonishing diversity of fungal species but studies have tended to concentrate on the identification of secondary metabolites rather than their ecological role (Bugni & Ireland, 2004). Some fungal communities associated with sponges have been described (Gao, Li, et al., 2008), but the type of relationship between the two organisms has not been determined. Fungal pathogens of marine animals or algae were reported and were able to

cause severe infections (Hyde, Jones, et al., 1998). In deep-sea ecosystems, the yeast-like fungus *Exophiala* was identified as the causative agent of mass mortalities in deep-sea endemic mussels (Van Dover, Ward, et al., 2007). Some other marine fungi are described, as skeletal-components of healthy, partially dead, and diseased corals (Ravindran, Raghukumar, et al., 2001). *A. sydowii* was found to cause an epizooty among sea fan corals (Alker, Smith, et al., 2001). *Scolecobasidium* sp. was the causative agent of necrotic patches on five different corals, *Porites lutea*, *Porites lichen*, *Montipora tuberculosa*, *Goniopora* sp., and *Goniastra* sp. (Raghukumar & Raghukumar, 1991). A similar fungus was retrieved in deep-sea corals but no infections were reported (Burgaud, Le Calvez, et al., 2009). Golubic, Radtke, et al. (2005) observed that fungi were able to penetrate the live tissues of different coral species and feed on organic matter using digestive enzymes.

Knowledge of marine fungi based on their function is still in its infancy. At present, only the well-known functions are observed although some evidence (See "Fungal metagenomics" in this chapter) suggests more diversified roles.

Culture and Isolation Techniques Used to Study Marine Fungi in Past Decades and at Present

Deep-sea conditions are different from those typically applied in laboratories. One of the major challenges in marine mycology is to isolate the fungal communities that are present and active in this extreme ecosystem characterized by a set of environmental restrictions such as high and low temperatures and high hydrostatic pressures. The culture media typically used in marine mycology are the same ones used to isolate terrestrial fungi and are composed of organic matter such as malt extract, potato-carrot, yeast extract, peptone, or glucose. The addition of antibiotics (chloramphenicol, streptomycin, penicillin, or vancomycin) is conventionally used to suppress bacterial growth to a generation time generally shorter. Zuccaro, Schulz, et al. (2003) compared culture-based and culture-independent methods and demonstrated a major dichotomy between strain collection and the molecular database generated. Most of the fungal isolates on the aforementioned media were thus presumed to be inactive or present in small amounts. Some exceptions occurred because *Corollospora angusta, Emericellopsis minima* (Zuccaro, Schulz, et al., 2003) and *Acremonium fuci* (Zuccaro, Schoch, et al., 2008) were active marine fungi detected using both methods on seaweed samples.

Different strategies can be imagined to search for fungi in deep-sea habitats. As proposed by Kohlmeyer and Kohlmeyer (1979), a low throughput method based on the observation of fungal structures can be used directly on marine substrates to characterize active marine fungal communities growing in situ. Direct detections using the specific fluorescent stain Calcofluor or

immunofluorescence using antibodies have also been applied to reveal *Aspergillus* strains in deep sediments from the Indian Ocean (Raghukumar, Raghukumar, et al., 2004; Damare, Raghukumar, et al., 2006). An untargeted approach allows the characterization of culturable fungal communities present on the surface or inside a marine substrate. The low availability of nutrients in deep-sea sediments requires the use of one-fifth-strength media to simulate the relative oligotrophic in situ conditions (Damare, Raghukumar, et al., 2006). Enrichment cultures were used to highlight fungi in marine sediments (Biddle, House, et al., 2005), deep-sea hydrothermal vents (Burgaud, Le Calvez, et al., 2009), and methane cold-seep sediments (Takishita, Yubuki, et al., 2007). Particle plating methods described by Bills and Polishook (1994) and also dilution plating along with pressure incubation were used to obtain fungal strains (Damare, Raghukumar, et al., 2006; Singh, Raghukumar, et al., 2010). Such methods have been used to isolate filamentous fungi and yeasts from marine sediments. To date, no endemic active marine fungi have been detected in deep marine sediments using a culture-based approach and only a minor fraction of the marine fungal community has been recovered by conventional selective media. Van Dover, Ward, et al. (2007) reported an epizootic event occurring on deep-sea mussels and hypothesized that the detected fungi could be facultative parasites or opportunistic pathogens. Chytrids were revealed using molecular methods in deep-sea environments (Bass, Howe, et al., 2007; Le Calvez, Burgaud, et al., 2009) with close representatives defined as parasites. The putative prevalence of parasitic fungal forms in deep-sea environments may explain the gap between cultural and molecular methods but also drastically hinders the experimental strategy to be used for cultures.

Depending on the nature of the samples, the estimated cultivation efficiency with standard techniques is between 0.001 and 1 percent (Amman, Ludwig, et al., 1995). Thus, the microorganisms cultured do not reflect the diversity present in the ecosystem studied. The evolution of innovative culture techniques mimicking deep-sea conditions will probably enhance the number of deep marine fungal strains that have currently been identified only by applying molecular methods. In the future, to improve cultivation efficiency one will need to consider (a) the chemistry of natural habitats, (b) the natural biotic and abiotic interactions, and (c) cell-to-cell communication (Alain & Querellou, 2009). One way to preserve endogenous cell-to-cell communication mechanisms is to process cultures in gel micro-droplets using microencapsulation. Gel micro-droplets were incubated under a constant stream of depleted medium nutrients and then sorted by flow cytometry. This approach preserves an exchange of metabolites and signals between cells and can provide more than 10,000 bacterial and fungal isolates from environmental samples (Zengler, Walcher, et al., 2005). The extinction-dilution technique in liquid medium described by Stingl, Tripp, et al. (2007) appears as another effective method to feed culture collections.

Uncultured Fungi and Molecular Approaches

Molecular approaches enable the description of an extended diversity because they allow uncovering sequences from species not yet cultured or the distinction between cryptic species. The description of fungal diversity has been enhanced by molecular techniques (e.g., Richards & Bass [2005]). Recently, the *Cryptomycota* (Jones, Forn, et al., 2011a) was described mainly from molecular data and characterized as a new phylum composed of one known genus (*Rozella*) and environmental sequences from different habitats including marine systems and more precisely deep-sea sediments (Nagano, Nagahama, et al., 2010; Nagahama, Takahashi, et al., 2011) and microaerobic seawater (Stoeck & Epstein, 2003).

Regarding marine systems, the presence of fungi was initially serendipitously detected in microeukaryote inventories and the fungal diversity described was low, that is, one to six OTUs (López-García, Rodríguez-Valera, et al., 2001; Stoeck & Epstein, 2003; Stoeck, Hayward, et al., 2006) with some exceptions of dominance in deep-sea sediments (Takishita, Tsuchiya, et al., 2006; Edgcomb, Beaudoin, et al., 2011). However, such studies were targeting a wide spectrum of microorganisms, mainly protists. The description of fungi and the weight of fungal communities may have been biased because of their poor representation in the Eukaryota biomass at the sites examined (Massana & Pedrós-Alió, 2008). This bias is also enhanced by the low sequencing effort in clone libraries, which rarely attains saturation (Edgcomb, Kysela, et al., 2002; Richards & Bass, 2005; Stoeck, Hayward, et al., 2006). Finally it has been shown that the described diversity mainly depends on the primers used. Indeed, an overlap of only 4 percent was observed between species lists obtained with three different primer pairs used (Stoeck, Hayward, et al., 2006). Another issue is contamination by organisms from other habitats such as autotrophs from the upper seawater columns. Indeed, comparison of the SSU rRNA gene using DNA and RNA-based approaches has revealed an absence of active autotrophs in deep-sea sediments even though their DNA sequences were retrieved from the same samples (Edgcomb, Beaudoin, et al., 2011). On the contrary, fungal sequences were abundant with *Basidiomycota* representing most of the microeukaryotic diversity described using DNA and cDNA. The repeated occurrence of fungal sequences in microeukaryote inventories motivated the use of specific fungal primers rather than universal eukaryotic primers to describe fungi in oceans.

The molecular approaches specifically designed to assess marine fungal diversity were focused on the 18S rRNA gene (Bass, Howe, et al., 2007; Le Calvez, Burgaud, et al., 2009; Nagahama, Takahashi, et al., 2011) but also internal transcribed spacers (Lai, Cao, et al., 2007; Nagano, Nagahama, et al., 2010), 5.8S (Nagano, Nagahama, et al., 2010), and 28S (Nagahama, Takahashi, et al., 2011). Most of the retrieved sequences show affinities with upper fungi.

To date, 35 OTUs belonging to undescribed fungal species have been retrieved (Richards, Jones, et al., 2012). Novel marine fungal OTUs from basal lineages (*Chytridiomycota, Blastocladiomycota,* and *Cryptomycota*) represent one third of the 35 OTUs retrieved and reveal a greater divergence from terrestrial fungal sequences than other OTUs (Richards, Jones, et al., 2012). One explanation could be the bias resulting from the primer pairs used as shown previously for eukaryote marine inventories. Another could be the global knowledge of the diversity of basal lineages, whatever the ecosystem. This has a direct impact on the sequences of basal lineages present in the molecular databases such as GenBank.

Molecular approaches are cogent methods to describe fungal diversity in deep-sea ecosystems. The continual recovery of novel OTUs using different kind of samples and different primer pairs reinforce the hypothesis that marine fungal diversity is higher than previously thought (Edgcomb, Kysela, et al., 2002; Stoeck, Hayward, et al., 2006; Jebaraj, Raghukumar, et al., 2010). Among marine fungi, basal lineages appear more abundant than expected. Use of specific primers in both marine and terrestrial ecosystems will help clarifying their diversity and ecological role.

Required Adaptation to Deep-Sea Conditions: The Hydrostatic Pressure

Many factors govern biodiversity in the oceans (e.g., temperature, salinity, pH, and nutrient availability). The majority of the biosphere is occupied by oceans with an average depth of 3800 m (Abe, 2007) indicating, as a Gaussian distribution, that most of the biosphere is subjected to a pressure of 38 megapascals (MPa), that is, 380-fold higher than the atmospheric pressure (0.1 MPa). Hence, the hydrostatic pressure appears as a key physical parameter, even defined as the most unique physical parameter, in the dark cold abyss (Lauro & Bartlett, 2008). Deep-sea microorganisms can be defined as piezophilic, piezotolerant or piezosensitive: (a) piezophiles display optimal growth rates at pressures higher than 0.1 MPa and below 60 MPa whereas hyperpiezophiles are defined as showing optimal growth at a pressure above 60 MPa, (b) piezotolerant display an optimal growth rate at pressures between 0.1 and 50 MPa but are able to grow at a pressure above 50 MPa, and (c) piezosensitives are sensitive to elevated hydrostatic pressures (Abe & Horikoshi, 2001; Bartlett, 2002).

Although many piezotolerant prokaryotes (Wang, Wang, et al., 2008), piezophilic prokaryotes (Kato, Sato, et al., 1995; Bernhardt, Jaenicke, et al., 1988), and even piezophilic hyperthermophilic archaeon (Zeng, Birrien, et al., 2009) have been isolated and characterized, only a few studies are focusing on the adaptation of fungi to hydrostatic pressure exception made for the model yeast *Saccharomyces cerevisiae* that has been intensively analyzed and characterized as piezosensitive (Fig.15.2).

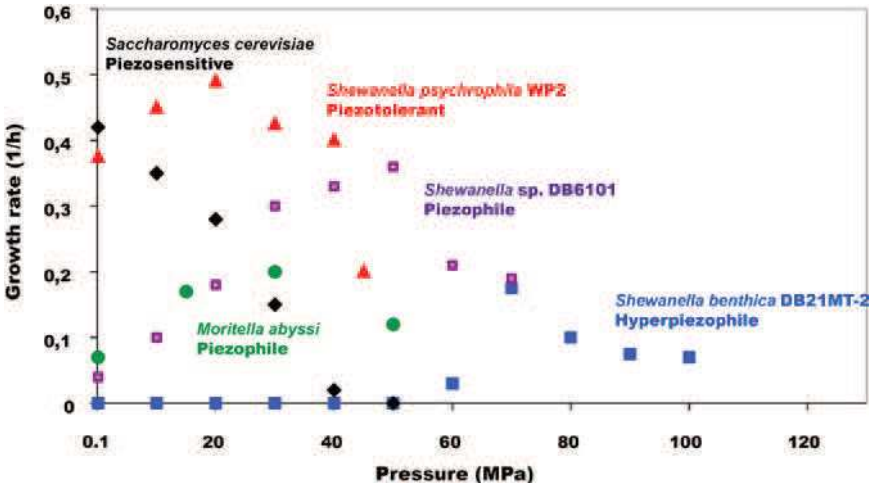

Figure 15.2 Growth rate of different microorganisms under pressure (Abe & Horikoshi, 1995; Kato, Sato, et al., 1995; Kato, Li, et al., 1998; Wang, Wang, et al., 2008; Xu, Nogi, et al., 2003).

Kohlmeyer and Kohlmeyer (1979) indicated that a simple method to obtain indigenous deep-sea fungi is to search for them directly on substrates that had been submerged in the deep sea at known depths. Wood infested by marine borers, chitin of hydrozoa, and calcareous shells appeared as ecological niches for fungal decomposers (Kohlmeyer, 1977; Raghukumar & Raghukumar, 1998). Five indigenous deep-sea fungi, *Abyssomyces hydrozoicus*, *Bathyascus vermisporus*, *Oceanitis scuticella*, *Allescheriella bathygena*, and *Periconia abyssa*, were respectively visualized directly on hydrozoa (1) at 600-m depth or wood (4) at depths below 1600 m but were not cultured. Ascospores of *A. hydrozoicus* and *O. scuticella* have mucilaginous appendages appearing as floating and attachment devices indicating an adaptation to marine habitats but not to deep high-pressurized environments. Dupont, Magnin, et al. (2009) collected wood fragments in the Vanuatu archipelago at depths between 100 and 1200 meters. Excised sporocarps from small twigs and sugar cane debris were analyzed using molecular methods and microscopy (i.e., scanning electron microscopy [SEM] and transmission electron microscopy [TEM]). The occurrence and wide distribution of *O. scuticella* in deep oceans was confirmed and a novel fungal pyrenomycete named *Alisea longicolla* was described and placed in the *Halosphaeriaceae,* the largest and most diverse lineage of marine ascomycetes to date (Sakayaroj, Pang, et al., 2011). Although fungal spores were plated on 2% malt extract agar or 2% water agar at 50° F (10° C), no growth was ever visualized (Dupont, Magnin, et al., 2009), which could be the result of the absence of elevated hydrostatic pressure

during culture or the short incubation time. As noted by Kohlmeyer (1969), after 13 months immersion of wood panels at 2000-m depth, only sterile hyphae were observed. Ascocarps were only visualized after 2 to 3 years. Other species of *Bathyascus* and *Periconia* were respectively isolated from shallow water marine habitats (Nambiar, Raveendran, et al., 2008) or found in soil or marine habitats on wood or sea hares (Nambiar, Raveendran, et al., 2008; Usami, Ichikawa, et al., 2008). Such species are good candidates to assess growth rate and morphological adaptations between shallow-waters and deep-sea representatives. Calcareous shells of animals recovered at 860- and 965-m depth were examined for endolithic fungi (Raghukumar & Raghukumar, 1998), and some stained fungal borers were visualized after dissolution of carbonate shells, suggesting an ecological role of decomposers. Epifluorescence microscopy can also be applied to sediment samples using Calcofluor staining or an immunofluorescence technique, for example, antibodies raised for *Aspergillus terreus* commonly retrieved in Central Indian Basin sediments (Damare, Raghukumar, et al., 2006). Fungi were detected in low abundance when such techniques were used. Recently, Damare and Raghukumar (2007) hypothesized that this low abundance was the result of macroaggregation of fungi in deep-sea sediments because ethylenediaminetetraacetic acid (EDTA) treatment of sediment particles released highest fungal particles. The authors suggested that fungal biomass was much higher than expected in sediments and might be involved in humic aggregate formation in deep-sea sediments. Such techniques may be enhanced with other methods classically used to reveal and count prokaryotes in deep sediments using EDTA, Tween 80, sodium-pyrophosphate, methanol, and ultrasonic treatment with or without a carbonate dissolution step with an acidic acetate buffer (Kallmeyer, Smith, et al., 2008) to reveal all the fungal elements in sediments.

A culture-based approach on calcareous shells led to the isolation of filamentous fungi commonly retrieved from terrestrial environments, for example, *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp. (Raghukumar, Raghukumar, et al., 1992; Raghukumar & Raghukumar, 1998). Although *Aspergillus restrictus* was able to penetrate shells and release calcium, these studies were unable to allow precise strain definition because only two pressures were tested (0.1 and 10 MPa). However, based on the current definition of Abe and Horikoshi (2001), such terrestrial strains are definitely piezosensitive. The same culture-based approach led to the isolation of numerous *Aspergillus* sp. strains (Damare, Raghukumar, et al., 2006; Damare, Nagarajan, et al., 2008) or a wider diversity with *Aspergillus* sp., *Cladosporium* sp., *Exophiala* sp., and *Acremonium* sp. (Singh, Raghukumar, et al., 2010) from deep-sea sediments but only piezosensitive fungi were retrieved. By testing several pressures, Lorenz and Molitoris (1997) defined precisely strains such as the piezosensitive facultative marine yeasts *Rhodotorula rubra*, *Debaryomyces hansenii*, and *Rhodosporidium sphaerocarpum* and thus

respected the postulate of Kohlmeyer and Kohlmeyer (1979:42) that "tests for tolerance of high pressures … can indicate whether the isolated fungal species are indigenous deep-sea forms or aliens from other habitats." The only issue remains the incubation time because hyphae may need several months to grow and reproductive forms several years. An in situ hybridization technique may be applied on fungal strains growing under different hydrostatic pressures to assess and quantify targeted-rRNA that can be correlated to the cellular ribosome content which, in turn, reflects the relative activity of cells (Poulsen, Ballard, et al., 1993; Binder & Liu, 1998; Daims, Lücker, et al., 2006).

High-pressure is mainly used by microbial ecologists to understand the ecological role of marine microorganisms but also by food technologists to inactivate microorganisms and by biotechnologists to enhance the productivity of bioprocesses (Follonier & Zinn, 2012). The effects of high-hydrostatic pressure on cells and cellular components are diverse: (a) pressure-sensitive lipids modifying the fluidity, permeability, and functioning of cell membranes; (b) pressure-sensitive proteins affecting multimer association and stability, (c) pressure-stabilized DNA hydrogen bonds affecting the replication and transcription steps requiring formation of single-strand DNA, and (d) loss of flagellar motility (Bartlett, 2002; MacGregor, 2002; Winter & Jeworrek, 2009; Oger & Jebbar, 2010; Follonier & Zinn, 2012). Piezophiles adapted to high pressures display specific adaptations, as compared to piezosensitive strains such as *Escherichia coli*, showing pressure-sensitive processes such as motility (Meganathan & Marquis, 1973), cell division leading to filamentation under pressure (Zobell & Cobet, 1964), growth, DNA replication, and translation (Yayanos & Pollard, 1969).

The model yeast *S. cerevisiae* has been widely analyzed to describe cellular responses to high pressure, named piezophysiology (Abe, 2004). *S. cerevisiae* is piezosensitive, meaning that no growth occurs at pressures higher than 50 MPa because of disruption of the microtubules, actin filaments, and nuclear membranes (Kobori, Sato, et al., 1995). Stress treatment of yeast cells with temperature (heat-shock or cold shock), ethanol (6%), or H_2O_2 (0.4 M) increases piezotolerance (Palhano, Gomes, et al., 2004). This cross-protection is mainly explained by heat shock protein (HSP) synthesis and enhancement of trehalose metabolism (Singer & Lindquist, 1998) acting as a whole stress response. The frontiers of basic metabolism are apparently not defined since Abe and Horikoshi (1998) and Abe (2004) argue that low cytoplasmic pH slows down glycolysis and to some extent stops ethanol fermentation around 50 MPa, whereas Picard, Daniel, et al. (2007) reported an ethanolic fermentation of 30 percent at 65 MPa that was definitely inhibited at 87 MPa. Studies of this model yeast may provide clues about the adaptation of fungi to high-pressure conditions. Even if *S. cerevisiae* is piezosensitive, this yeast is able to modify its membrane composition to tolerate high-hydrostatic pressure but only under short-term treatment. After

30 minutes at 200 MPa, *S. cerevisiae* up-regulates *ole1* gene expression to increase the proportion of unsaturated fatty acids (Fernandes, Domitrovic, et al., 2004) and prevents the hydrostatic pressure effect by increasing membrane fluidity. *S. cerevisiae* also upregulates *erg25* gene expression involved in ergosterol biosynthesis, and it is hypothesized that ergosterol may be an important protector of the cell membrane (Fernandes, 2004). Such adaptations may explain the presence of *S. cerevisiae* at 250- to 500-m depth in the water column (Bass, Howe, et al., 2007).

Filamentous fungi cultured under elevated hydrostatic pressure display abnormal structures; for example, *Aspergillus* from deep-sea sediments has altered reproduction forms with long hyphae instead of conidial heads or even hyphal swellings (Raghukumar, Raghukumar, et al., 2004; Damare & Raghukumar, 2007). Morphological anomalies occurred on piezosensitive microorganisms such as *Escherichia coli* that forms filament under increased hydrostatic pressure (Zobell & Cobet, 1964). Hydrostatic pressure was found to dissociate FtsZ protein polymers that normally play the most important role in the cell-division process (Ishii, Sato, et al., 2004). More data must be gathered to understand the abnormal morphologies of *Aspergillus* and to find out whether some yeasts become filamentous under high-pressure conditions.

Some molecular trends are directly correlated to piezophily. The occurrence of elongated helices in the 16S rRNA genes was mostly detected in piezophiles (Lauro, Chastain, et al., 2007) indicating a piezospecificity. A 454-pyrosequencing metagenomic data set from the Puerto Rico Trench at 6,000-m depth demonstrates that this deep microbial community possesses large genomes enriched in signal transduction, transcriptional regulators, and transporter mechanisms (Eloe, Fadrosh, et al., 2011). But, to the current knowledge, no universal genetic marker of piezophily is yet known and metagenomics are still unable to clearly segregate piezosensitive, piezotolerant, and piezophiles.

Fungi in Deep-Sea Sediment and Hydrothermal Ecosystems: Toward a New Evolutionary Paradigm

The deep subsurface biosphere and hydrothermal ecosystems represent large biomes on Earth characterized by a set of extreme conditions (darkness, low or high temperatures, anoxia, elevated hydrostatic pressure, relative oligotrophy or organic-richness). Whereas hydrothermal vent ecosystems have been exhaustively characterized by geologists, chemists, biologists, and microbiologists since 1979, only recent studies have estimated that the deep subseafloor biosphere comprises a significant fraction of the Earth's microbial biomass. However, the fungal communities occurring in those extreme ecosystems remain mostly undescribed.

Hydrothermal Ecosystems: Ecological Niches for Fungi?

Life on the seafloor of deep oceans relies on organic matter mainly sinking from the upper layers. Productivity in these environments is generally low and abyssal ecosystems are considered as deserts with a biomass of about 1 $g.m^{-2}$ (Jahnke & Jackson, 1992). In contrast, deep-sea hydrothermal ecosystems are considered as hot spots of productivity on Earth with a biomass exceeding 50 kg/m^{-2} (Desbruyeres, Almeida, et al., 2000). The discovery of hydrothermal sites ranging from shallow waters—Vulcano active site in Italy (Gugliandolo & Maugeri, 1993)—to 4960-meter depth (Connelly, Copley, et al., 2012) have totally refined the biogeosciences. Hydrothermalism is directly correlated to the volcanic activities occurring in different parts of the oceanic ridges (Fig. 15.3). The hydrothermal fluids emitted by smokers result from the heating of seawater, in contact with the magma mantle, and are enriched with reduced compounds, such as hydrogen sulfide (H_2S), methane (CH_4), or carbon dioxide (CO_2) (Jannasch, 1989; Karl, 1995). The primary production on which the biological communities in these ecosystems depend is finely tuned by chemoautotrophic prokaryotes, which constitute the basis of the food web (Jannasch, 1989; Fisher, 1995). These microorganisms harvest their energy by oxidizing the reduced compounds emitted by the fluids (Van Dover & Fry, 1994; Childress, Fisher, et al., 1991). The energy released can be used to convert mineral carbon to organic carbon that may subsequently be used by a large panel of heterotrophic macroorganisms (mussels, shrimps, clams, tubeworms).

It is well established that the hydrothermal vent biosphere harbors a high microbial biomass mainly composed of prokaryotes. The occurrence of fungal sequences in hydrothermal vent environments of the Guaymas Basin (Gulf of California) was assessed about a decade ago (Edgcomb, Kysela, et al., 2002). Fungi were exclusively retrieved from the sediment-seawater interface layer and not from deeper cores at higher temperature (149° F [65° C]). Using similar approaches, the incidence of fungi in hydrothermal vents was confirmed and a wider diversity was revealed at the Mid-Atlantic Ridge and East Pacific Rise hydrothermal sites (López-García, Philip, et al., 2003; López-García, Vereshchaka, et al., 2007; Bass, Howe, et al., 2007; Le Calvez, Burgaud, et al., 2009; Sauvadet, Gobet, et al., 2010). The group "hydrothermal and/or anoxic marine yeasts" appeared as the most consistently detected fungi in clone libraries and the initial occurrence of chytrids in deep-sea ecosystems was reported. The first culture-based approaches on samples collected from deep-sea environments in the Mid-Atlantic Ridge and in the North-West Pacific Ocean led to the isolation of yeasts composed of known and previously undescribed species (Gadanho & Sampaio, 2005; Nagahama, Hamamoto, et al., 2006; Burgaud, Arzur, et al., 2011). Deep-sea hydrothermal yeasts were frequently found associated with endemic fauna, which raised questions regarding their ecological role (Burgaud, Arzur, et al., 2010). Such yeasts may be facultative parasites or

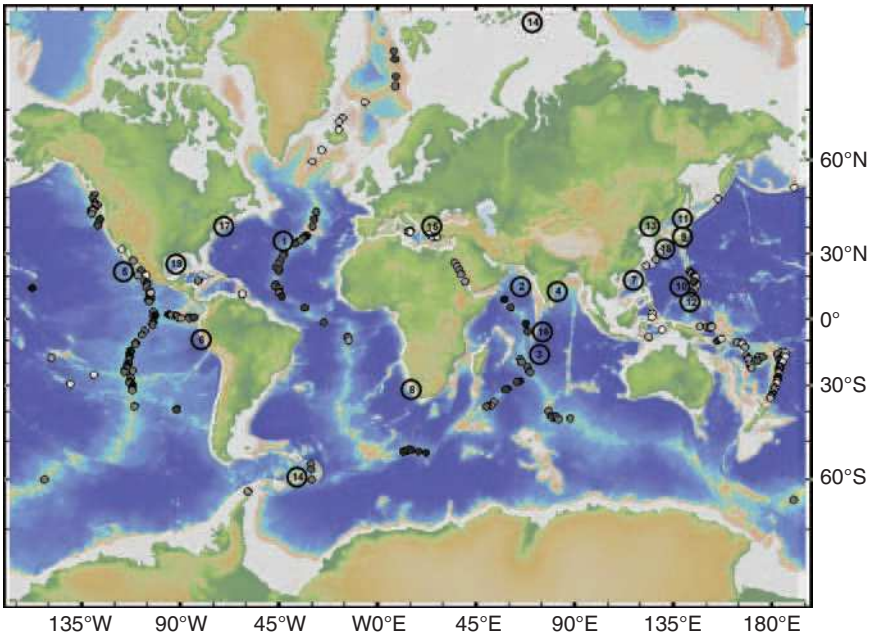

Figure 15.3 Location of known hydrothermal sites with reported depth: 0–1000 m, 1000–2000 m, 2000–3000 m, >3000 m) and location of sediment sampling sites and associated studies dealing with fungal communities. 1. Mid-Atlantic Ridge: López-García, Philip, et al. 2003; Bass, Howe, et al. 2007. 2. Arabian Sea: Raghukumar & Raghukumar, 1998; Jebaraj & Raghukumar, 2009; Jebaraj, Raghukumar, et al., 2010. 3. Central Indian Basin: Damare, Raghukumar, et al., 2006; Singh, Raghukumar, et al., 2010; Singh, Raghukumar, et al., 2011; Singh, Raghukumar, et al., 2012. 4. Bay of Bengal: Raghukumar & Raghukumar, 1998; Das, Lyla, et al., 2009. 5. Guaymas Basin: Edgcomb, Kysela, et al., 2002. 6. Peru margin & Peru trench: Edgcomb, Beaudoin, et al., 2011. 7. South China Sea: Lai, Cao, et al., 2007. 8. St Helena Bay: Mouton, Postma, et al. 2012. 9. Sagami Bay: Nagahama, Hamamoto, et al., 2001; Nagahama, Hamamoto, et al., 2003; Takishita, Yubuki, et al., 2007; Nagahama, Takahashi, et al., 2011. 10. Yap Trench: Nagahama, Hamamoto, et al., 2006. 11. Japan Trench: Nagahama, Abdel-Wahab, et al., 2008. 12. Mariana Trench: Takami, Inoue, et al., 1997; Nagano, Nagahama, et al., 2010. 13. East Sea: Park, Park, et al., 2008. 14. Arctic and Southern Ocean: Pawlowski, Christen, et al., 2011. 15. Sea of Marmara: Quaiser, Zivanovic, et al., 2011. 16. Chagos Trench: Raghukumar, Raghukumar, et al., 2004. 17. Cape Cod, Massachusetts: Stoeck & Epstein, 2003. 18. Kagoshima Bay: Takishita, Miyake, et al., 2005. 19. Gulf of Mexico: Thaler, Van Dover, et al., 2012. *This figure was generated using GeoMapApp© and the actualized InterRidge Vents Database http://www.interridge.org/irvents/* (Beaulieu, 2010).

opportunistic pathogens of deep-sea animals (Van Dover, Ward, et al., 2007, Burgaud, Le Calvez, et al., 2009). This is emphasized by a recent report of fungal sequences in liquid from the pallial cavity of deep-sea hydrothermal bivalves (Savaudet, Gobet, et al., 2010). The yeasts and filamentous fungi retrieved (Burgaud, Le Calvez, et al., 2009) may also play a role in the decomposition of organic matter in such rich biomass environments.

Deep-Sea Sediments: A Tremendous Reservoir of Microbial Types including Fungi

Deep-sea sediments represent nearly two thirds of the Earth's surface and consequently the largest biome of the biosphere with a volume of 5.10^8 km^3 (*see* Fig. 15.3). However, the knowledge about this ecosystem and of the microbial-driven processes is still limited. Recent years have seen critical progress in exploration of the deep bottom mainly thanks to the International Ocean Drilling Program (IODP). The accessibility of deep-sea sediment samples provided crucial information about the microbial communities occurring and active at several hundred meters below the surface of the ocean floor (Roussel, Cambon-Bonavita, et al., 2008). Whitman, Coleman, et al. (1998) estimated that the marine subseafloor biosphere would host $3.5.10^{30}$ prokaryotic cells, but according to more recent estimates, the marine subseafloor biosphere would comprise 1/20th of all life on Earth or 5 to 15 percent of the Earth's microbial biomass (Kallmeyer, Pockalny, et al., 2009).

If the presence and activity of prokaryotes is becoming increasingly well documented, studies of eukaryotic diversity in deep-sea sediments remain sporadic. Pawlowski, Christen, et al. (2011) retrieved around 125,000 reads using 454-pyrosequencing on sediment samples. Among them, many phototrophs were detected, corresponding to dwelling organisms that sink to the bottom and can form up to 17 percent of the whole reads. Along with planktonic and metazoan reads, fungal sequences were retrieved but did not exceed 2 percent of the total assigned OTUs indicating their occurrence but at low abundance.

Takami (1999) isolated *Penicillium lagena* and *Rhodotorula mucilaginosa*, some ubiquist filamentous fungal and yeast strains, from Mariana Trench sediments at about 11,000-m depth. Several yeasts were isolated from deep marine sediments and described as novel taxa in the *Ascomycota* or *Basidiomycota* phyla (Nagahama, Hamamoto, et al., 1999; Nagahama, Hamamoto, et al., 2001; Nagahama , Hamamoto, et al., 2003; Nagahama, Hamamoto, et al., 2006; Nagahama, Abdel-Wahab, et al. 2008). The specific occurrence of fungi in deep-sea sediments is well documented for the Central Indian Basin (Damare, Raghukumar, et al., 2006; Das, Lyla, et al., 2009; Singh, Raghukumar, et al., 2010; Singh, Raghukumar, et al., 2011; Singh, Raghukumar, et al., 2012). Several filamentous fungi and yeasts were detected and were able to grow under elevated hydrostatic pressure. Ascomycetes were mainly represented by filamentous fungi and basidiomycetes by unicellular yeast forms (Singh, Raghukumar, et al., 2010). Jebaraj and Raghukumar (2009) isolated filamentous fungi and yeasts from marine sediments and indicated that several species of fungi were able to grow at close-to-zero dissolved oxygen levels and were actors in denitrification processes. This pattern was confirmed at St. Helena Bay where extracellular cellulases were synthesized by filamentous fungal isolates, thereby indicating their

putative role in detrital decay processes (Mouton, Postma, et al., 2012). These fungi, mostly affiliated to *Aspergillus* and *Penicillium*, play an active role in denitrification, co-denitrification, and ammonification processes in the nitrogen cycle in marine sediments.

Fungal molecular signatures have been detected from shallow (350 m) to deep (3011 m) marine sediments particularly rich in methane hydrates in the south of China Sea (Lai, Cao, et al., 2007). Phylogenetic analyses using internal transcribed spacers as barcode marker revealed a fungal diversity composed of *Phoma*, *Lodderomyces*, *Malassezia*, *Cryptococcus*, *Cylindrocarpon*, *Hortaea*, *Pichia*, *Aspergillus*, and *Candida*. Recently, Edgcomb, Beaudoin, et al. (2011) revealed that fungal communities were dominant among microeukaryotes in marine subsurface sediments of the Peru margin. Analyses of DNA and cDNA sequences allowed description of genetic and functional diversity and revealed mainly some uncultured basidiomycetes and a few ascomycetes. Molecular approaches have revealed a large fraction of uncultured deep-branching fungi in deep-sea methane cold-seep sediments (Nagahama, Takahashi. et al., 2011) and deep-sea sediments (Nagano, Nagahama, et al., 2010). The discovery of a novel basal fungal group in deep-sea ecosystems suggests the presence of a reservoir of previously unknown fungal biodiversity.

Pooling culture-dependent and culture-independent data provides confirmation that fungi are present and metabolically active in marine sediments and could play a major role in biogeochemical cycles in the deep biosphere. The fungal diversity retrieved in deep-sea sediments and hydrothermal ecosystems may give clues regarding fungal evolution and diversification of early lineages.

Fungal Diversity in Deep-Sea Sediments and Hydrothermal Ecosystems Tells Us a New Evolutionary Story

As shown, few studies have investigated the fungal diversity in deep-sea sediments and hydrothermal ecosystems, but the sequences retrieved in these inventories raise many questions regarding their activity, ecological role, and even the evolutionary story of the basal fungal lineages retrieved in those two extreme environments. Most of those sequences are clearly divergent from described species. Using sediment samples, sequences of three different basal phyla were detected. Sequences of *Cryptomycota* were obtained from samples collected in the Mariana Trench (Nagano, Nagahama, et al., 2010). In the same way, organisms belonging to early diverging lineages appear to dominate the fungal communities retrieved in the methane cold seeps in the Sagami Bay (Nagahama, Takahashi, et al., 2011). Among them, five OTUs form a new phylogenetic clade close to *Blastocladiomycota* (Richards, Jones, et al., 2012) even if the maximal identity of this phylum was only of

91 percent. Moreover, sequences showing affinities with *Cryptomycota* were recovered from this kind of sediments. Finally, *Chytridiomycota* sequences were obtained from a methane cold seep located in the Gulf of Mexico (Thaler, Van Dover, et al., 2012). Regarding hydrothermal vents, only *Chytridiomycota* sequences have been harvested and these were found associated with *Bathymodiolus azoricus* mussels in hydrothermal vents at 860 - and 1,700-m depth (Le Calvez, Burgaud, et al., 2009). None of them present close phylogenetic relationships with described species. These results lead to the hypothesis of a diversification of fungi in deep-sea hydrothermal ecosystems. Hydrothermalism was likely mundane when fungi emerged during Precambrian (Robert & Chaussidon, 2006). Given the molecular clock estimates, the emergence and diversification of fungi in marine environments before land colonization is a reasonable hypothesis (Le Calvez, Burgaud, et al., 2009). And the flagellum loss (Liu, Hodson, et al., 2006) or losses (James, Kauff, et al., 2006) could be regarded as a possibility of a better dispersion and resistance of spores (Le Calvez, Burgaud, et al., 2009). Alternatively, the fungal emergence in freshwater ecosystems has been suggested (Richards, Jones, et al., 2012). The newly detected clades are initiating a new era in the understanding of fungal evolution and diversification of the early diverging lineages. Using high-throughput sequencing of genetic markers on several aquatic and marine ecosystems will certainly provide insights and settle this unsolved question.

Fungal Metagenomics: Predictions of Functions and Biotic Interactions

To date little is known about fungal diversity in hydrothermal ecosystems and the functions exerted are enigmatic. It is now possible to predict metabolic pathways from genes and thereby deduce lifestyles by a metagenomic approach (for review see Vandenkoornhuyse, Dufresne, et al., 2010), the metagenome being defined as the sum of the genomes of all organisms living in a given environmental sample. By applying this approach to a chosen sample the ecological functions of one fungal organism was aimed to be predicted in its habitat belonging to an unknown deep branching *Chytridiomycota* lineage (phylotype 1 in Le Calvez, Bugaud, et al., 2009). The sample processed corresponded to a biofilm on the outside of a *Bathymodiolus azoricus* shell. The working hypothesis was that fungi outside the mussel were heterotrophic.

Three 454-pyrosequencing runs were performed. The sequencing effort was checked to make sure it was sufficient to properly analyze the metabolic properties of the sample by applying genome recruitment tests (i.e., coverage tests from reference microorganisms in the metagenome). The metagenome consisted of a large majority of bacterial sequences.

Prediction of Fungal Functions in Hydrothermal Ecosystems from the Metagenome

Some 254 contigs were obtained (average length of 3400-base pairs). Approximately three fourths of those data contained metabolic information that allows producing hypotheses about fungal lifestyles and their ecological role in this ecosystem.

Amino Acid Metabolism Protein-coding genes involved in the synthesis and degradation of most amino acids were harvested: cysteine, lysine, histidine, glutamate, tyrosine, glycine, serine, threonine, leucine, valine, isoleucine, alanine, aspartine, and phenylalanine. Thus, it can be suggested that a pathogenic lifestyle could be rejected.

Carbohydrates and Energy Metabolism Protein-encoding genes involved in the pentose phosphate pathways, citrate cycle, and glycolysis were predicted from the annotation. Enzymes were found for the aerobic carboxylation of glucose, as well as enzymes involved in the anaerobic decarboxylation of pyruvate, a process characteristic of yeast-like metabolism. Thus, the studied fungus within the metagenome might be able to ensure both yeast-like and filamentous-like metabolism. Because enzymes involved in oxidative phosphorylation were predicted, the hypothesis of aerobic metabolism is reinforced. Signatures of genes involved in glycogen, formate, and pyruvate catabolism were also predicted. Surprisingly, no heterotrophic gene signatures were found, such as genes encoding for chitinases or glucosidases (typically found in fungal organisms). This might stem from the limited number of fungal contigs detected in the data set: the main carbon source of these organisms remains to be elucidated.

Along with a fungal antibiotic biosynthesis prediction, bacterial sequences involved in penicillin degradation and bacterial penicillin-binding proteins were found, suggesting biotic interactions between fungi and bacteria. It was suggested that the fungus produces and emits antibiotics into the environment (allelopathy mechanism) to improve its ability to compete in colonizing the ecological niche or habitat.

Global Analyses

Predictions of Autotrophic C Fixation Carbon assimilation in deep hydrothermal ecosystems relies on chemolithoautotrophy (Pimenov, Lein, et al., 2000). Many reads in the metagenome were assigned to ribulose biphosphate carboxylase (RuBisCo) that can fix CO_2, with water to form two molecules of phosphoglycerate from the substrate. Most autotrophic microorganisms,

including hydrothermal-living organisms, use the Calvin Benson Cycle to assimilate CO_2 (Childress & Fisher, 1992). All the known enzymes in this cycle were found.

Methane Assimilation At the hydrothermal site studied, Lucky Strike, methane is emitted by the smokers within fluids (Pimenov, Lein, et al., 2000), and 115.2 µM has been detected (in the fluid) at this particular site (Desbruyères, Biscoito, et al., 2001). Methanotrophy is therefore supposed to be common.

As expected, the analyses of the metagenome data set revealed enzymes involved in methanotrophy. Enzymes transforming methane to methanol, methanol to formaldehyde, formaldehyde to formate, and formate to CO_2 were found.

As few reads were assigned to *Archaea,* it is hypothesized that, in this particular habitat, bacteria would mainly mediate methanotrophy. From our expert analyses, the two types of methanotrophic mechanisms (type I and type II) were hypothesized. However, only four reads were assigned to methane monooxygenase, the key enzyme driving the transformation of methane to methanol. There is thus discordance between the taxonomic analyses, which reveal a strong presence of methanogenic bacteria, and the functional predictions. A possible explanation could be the structure of this enzyme composed of three subunits, the genes of which could display considerable variation. Thus, there is a distortion between data to analyze and available sequences databases.

Concluding Thoughts and Future Directions

In this chapter the fact that fungi are living in the oceans and form diverse communities has been discussed. This feature clearly conflicts with the general dogma that fungi are exclusively terrestrial organisms. Recent data obtained in marine mycology introduce the possibility of a different story concerning fungal evolution. Loss of flagellum in higher fungi has been considered for decades as a signature of terrestrialization. An alternative hypothesis has been suggested recently by Le Calvez, Burgaud et al. (2009: 6416): "the loss of motile gametes in fungi was compensated for by the resistance and long-range dispersal of spores … and … this evolutionary innovation in eukaryotes should have led to colonization and longterm persistence in many new environments, including land. …" The better understanding of fungal kingdom evolution as a result of culture-independent molecular techniques has also led to the description of the *Cryptomycota* (Jones, Forn, et al., 2011a), most of which are found in marine environments. This newly described phylogenetic cluster led to the hypothesis of a new paradigm of fungal evolution (Jones, Richards, et al., 2011b) and suggested an earlier fungal emergence.

If it is now clear that fungi are living in oceans, the roles in ecological function remain poorly addressed. From comparisons with terrestrial species, it

would be expected fungi to play a key role in nutrients cycling in oceans. Original results from a metagenomic analysis of an environmental sample containing a single *Chytridiomycota* phylotype are presented herein. From fungal gene predictions and expert annotations of contigs, the aim was to understand how this fungus was living in a hydrothermal ecosystem. Unexpectedly, the absence of a genomic signature of heterotrophy was noted From the genes the possibility of antibiotics production was predicted and bacterial counterparts in the metagenome able to bind or degrade these antibiotics were found, thus producing close biotic interactions. From this, it is also clear that oceanic fungi must be regarded from a biotechnological point of view. It is possible to culture and isolate a fraction of these marine fungi as demonstrated by Burgaud, Le Calvez, et al. (2009).

Future fungal research should place a special focus on marine fungi depending on different perspectives. One primary issue would be to better understand fungal diversity and evolution. This would permit to redefine the evolutionary history of the Opisthokonts, especially how and when animals and fungi have diversified. A second issue would be to understand the fungal ecological functions and biotic interactions occurring in marine ecosystems. A key question here would be to address whether there is a parallelism in between the known ecological functions exerted in terrestrial and marine ecosystems. If fungi emerged and diversified in marine environments before colonizing land, as suggested by Le Calvez, Burgaud, et al. (2009), symbioses from mutualistic to pathogenic biotic interactions have developed. It is also possible that marine specific lifestyle(s) exist(s) for fungi. These aspects have been poorly documented to date but genomics, metagenomics, transcriptomics, and metatranscriptomics will be important strategies. A third issue includes all possible applied aspects related to the use of these marine fungi including enzymes, organic carbon transformation and energy, and drugs. This will likely relate to the ability to culture such fungi or to analyze the nucleic acids data displayed.

More widely, marine mycology should be regarded as an emerging field of research that will bloom and will be drained by ideas and knowledge from land mycology, theoretical ecology, and evolutionary paradigms.

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Comme nous l'avons vu dans cette partie, les champignons marins, qu'ils soient étudiés au niveau de sources hydrothermales ou de sédiments marins, présentent une large diversité encore que partiellement décrite. Dans la partie suivante, nous verrons que les technologies de séquençage à haut débit, sont à présent des techniques de choix pour décrire de façon plus exhaustive la diversité fongique génétique et fonctionnelle dans les écosystèmes marins.

1.4. Les nouvelles générations de séquençage

Le terme de nouvelle génération de séquençage ou NGS (Next Generation Sequencing) regroupe l'ensemble des technologies de séquençage développées depuis 2005. Ces nouveaux outils ont révolutionné la génomique en permettant de produire très rapidement, et à moindre coût, des quantités massives de données (Figure 18). Deux nouvelles générations de séquençage sont actuellement disponibles sur le marché, les technologies à haut débit dites de 2ème génération qui permettent de générer des dizaines de millions de séquences en parallèle et celles dites de 3ème génération permettant le séquençage d'une seule molécule d'ADN.

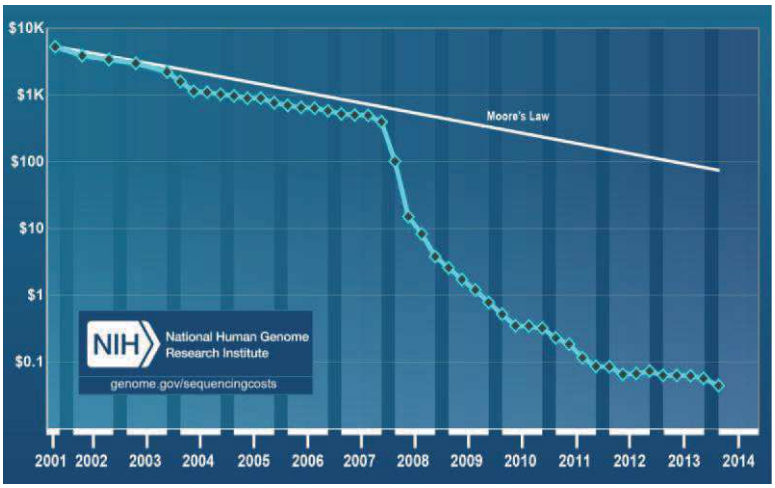

Figure 18 : Evolution du coût d'une megabase de séquence ADN en fonction des années comparativement à la loi de Moore.

1.4.1. Historique

La première technique de séquençage de l'ADN a été introduite par Sanger et son équipe en 1977. Prix Nobel de chimie en 1980, Frederick Sanger a été l'un des pionniers du séquençage de l'ADN en mettant au point une méthode moléculaire permettant d'accéder à la séquence de l'ADN grâce à l'incorporation de nucléotides particuliers, les

didésoxyribonucléotides. Ensuite, la technique de Sanger a été automatisée ce qui a permis la démocratisation du séquençage à partir des années 1990 (Hutchison, 2007). La version la plus moderne des séquenceurs capillaires est capable de séquencer jusqu'à 1 kb pour 96 migrations simultanément (Shokralla *et al.,* 2012). Cependant, jugée trop longue et particulièrement onéreuse la technique de Sanger a, depuis les années 2000, petit à petit laissé place à de nouveaux instruments de séquençage, notamment en génomique environnementale, permettant de produire plus rapidement, et à moindres coûts, des quantités massives de données.

1.4.2. Les technologies de deuxième génération

Apparues à partir de 2005, les méthodes de séquençage à haut débit permettent de produire plusieurs millions de séquences en parallèles. Ainsi, ces méthodes permettent d'obtenir un plus grand nombre de séquences, plus rapidement et pour un prix inférieur à la méthode de Sanger. Même si les techniques de nouvelles générations s'affranchissent le plus souvent de l'étape préalable de clonage des fragments ADN et des biais méthodologiques liés à cette technique, elles possèdent leurs propres limites. Basées sur une amplification par PCR, ces nouvelles techniques peuvent induire notamment la formation de chimères (Haas *et al.,* 2011). Formées à partir de deux ou plusieurs séquences biologiques reliés entres elles, les chimères sont rares dans les approches dites « shotgun » ou séquençage aléatoire mais sont relativement fréquentes dans les approches de « tag sequencing » (séquençage d'un marqueur moléculaire) où des séquences étroitement liées sont amplifiées. Ces erreurs ont pour conséquence une surestimation de la diversité présente au sein de l'échantillon biaisant ainsi la vision de la structure des communautés étudiées. Cependant, depuis 2005 les technologies NGS ont beaucoup évolué en termes de réduction des coûts, de qualité et de longueur des séquences produites (Figure 19). Le séquençage de deuxième génération permet aujourd'hui l'analyse de la diversité eucaryote en séquençant à haut-débit par exemple les régions hypervariables du gène codant pour l'ARNr 18S directement à partir d'échantillons environnementaux marins (*i.e*. Stoeck *et al.,* 2010; Pawlowski *et al*., 2011).

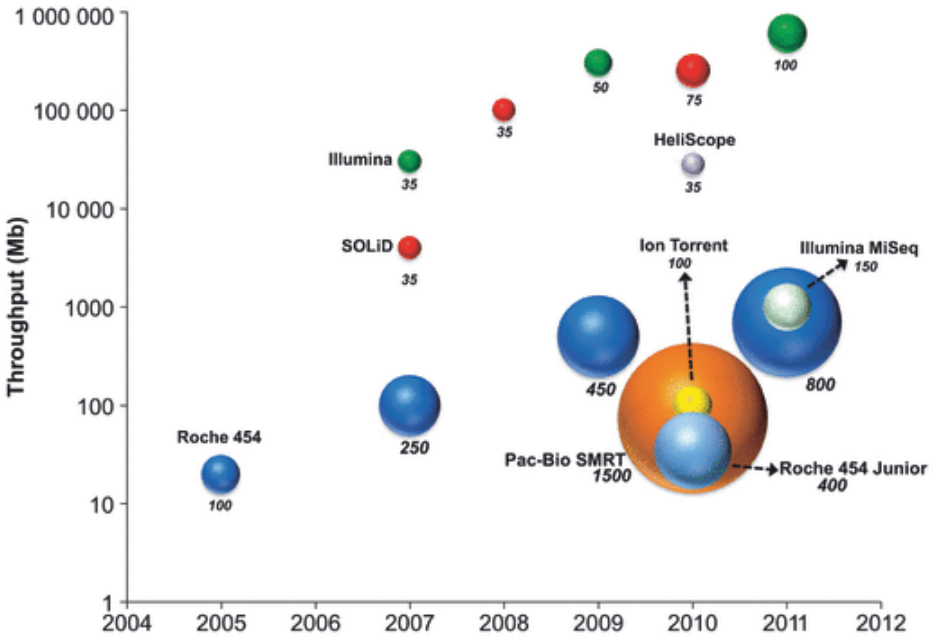

Figure 19 : Développement des NGS. D'après Shokralla *et al.* (2012)

Le diamètre de chaque rond représente la longueur des reads générés par la plateforme en pb. Les couleurs correspondent aux différentes plateformes.

Les techniques de séquençage de deuxième génération utilisent des méthodologies et des chimies différentes, mais nécessitent toutes la construction d'une librairie de séquences, qui consiste à fragmenter l'ADN génomique pour ensuite lier des adaptateurs à chacune des extrémités de ces fragments. Chaque fragment de la librairie est immobilisé sur un support solide et amplifié de manière à ce que chaque produit d'amplification soit séparé des autres. Le séquençage est ensuite effectué par plusieurs cycles de réactions enzymatiques et mesuré en temps réel (Glenn, 2011; Zhang *et al*., 2011). Les séquenceurs de deuxième génération sont représentés par le pyroséquençage 454 (Roche), les technologies Illumina HiSeq ou MiSeQ (Illumina), SOLiD (Applied Biosystems) ou Ion Torrent (Life Technologies). Dans ce manuscrit seront présentées uniquement les technologies 454 et Illumina, utilisées au cours de cette thèse.

1.4.2.1. Le pyroséquençage 454

Commercialisé depuis 2005, le pyroséquençage 454 a été la première plateforme de séquençage haut-débit sur le marché. La particularité de cette technique repose sur le fait que les brins d'ADN sont amplifiés par PCR en émulsion sur la surface de centaines de milliers de billes d'agarose, en simultané. A la surface de ces billes se trouvent des amorces complémentaires des séquences des adaptateurs qui ont été préalablement liés aux fragments à amplifier. L'amplification clonale produit alors des millions de copies de chaque fragment, au sein de microréacteurs (Figure 20). Chacune des billes est ensuite déposée sur une plaque en fibre optique contenant 1,6 million de puits. A la différence de la méthode de Sanger, le principe du pyroséquençage se base sur l'addition d'un seul nucléotide révélé en temps réel par détection de la luminescence. Un flux de nucléotides traverse la plaque en fibre optique et les nucléotides, sous forme de dNTP, sont ajoutés les uns après les autres. Si le nucléotide ajouté dans le milieu de réaction correspond à celui attendu par l'ADN polymérase, il est incorporé dans le brin en cours de synthèse et libère un pyrophosphate inorganique (PPi). L'ATP sulfurylase va utiliser le pyrophosphate relâché lors de la polymérisation pour générer de l'ATP. L'ATP formé est utilisé par la luciférase pour oxyder la luciférine en oxyluciférine, générant un signal lumineux proportionnel à la quantité d'ATP. L'apyrase quand à elle, dégrade les nucléotides non incorporés et l'excès d'ATP (Margulies *et al*., 2005). Bien que la substitution de base soit un événement rare, l'incorporation multiple de nucléotides est courante (Shokralla *et al.,* 2014). Les progrès en terme de chimie de séquençage, d''instrumentation et de logiciels ont permis de mettre au point le nouveau système GS FLX, de très haute précision (99,997%). Le GS FLX+ est le séquenceur de deuxième génération capable de produire les plus longues séquences (ou « reads) de 700 pb.

1.4.2.1. La technologie Illumina

Introduite en 2006, la technologie Illumina est basée sur un séquençage par synthèse couplé à une amplification par « bridge PCR » effectuée sur une surface solide appelée « flow cell ».

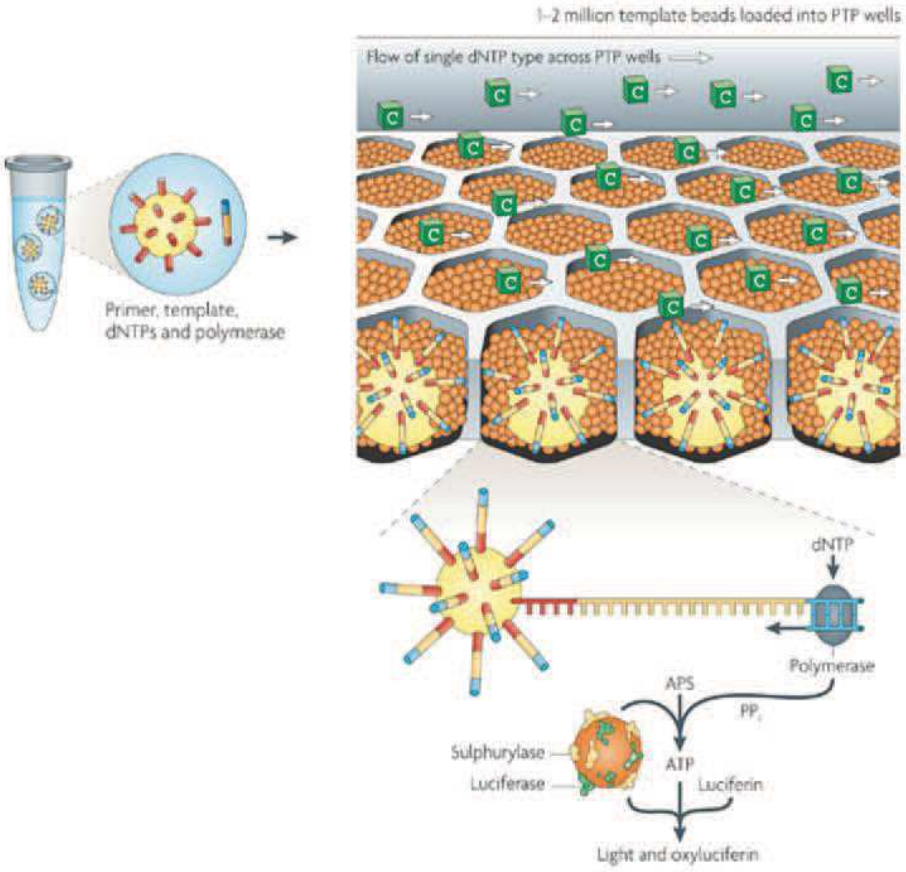

Figure 20 : Principe du pyroséquençage 454. D'après Metzker (2009)

Les fragments d'ADN de la librairie, pourvus d'adaptateurs spécifiques à leurs extrémités, vont venir s'hybrider aux oligonucléotides qui leurs sont complémentaires et liés de manière covalente à la surface de la lame. Après immobilisation des fragments ADN sur la surface de la plaque, le brin complémentaire de chaque fragment est synthétisé à l'aide d'une polymérase. L'amplification se déroule en ajoutant un mélange contenant les quatre nucléotides comportant chacun un terminateur de chaîne réversible marqué par un fluorophore spécifique de chaque nucléotide. Après excitation au laser, la fluorescence émise permet alors de détecter simultanément toutes les positions incorporées. Après clivage des terminateurs de chaîne, l'incorporation de la base suivante est possible. La lecture est effectuée ainsi cycle après cycle (Figure 21). La technologie Illumina permet l'acquisition en parallèle de plusieurs milliards de séquences de 150 à 2 x 300 pb selon la technologie utilisée. Bien que plus fiable que le pyroséquençage pour le séquençage de certains motifs comme les homopolymères ou les

régions répétées, l'utilisation de nucléotides modifiés réduit leur efficacité d'incorporation et engendre une augmentation de la fréquence des erreurs de substitution.

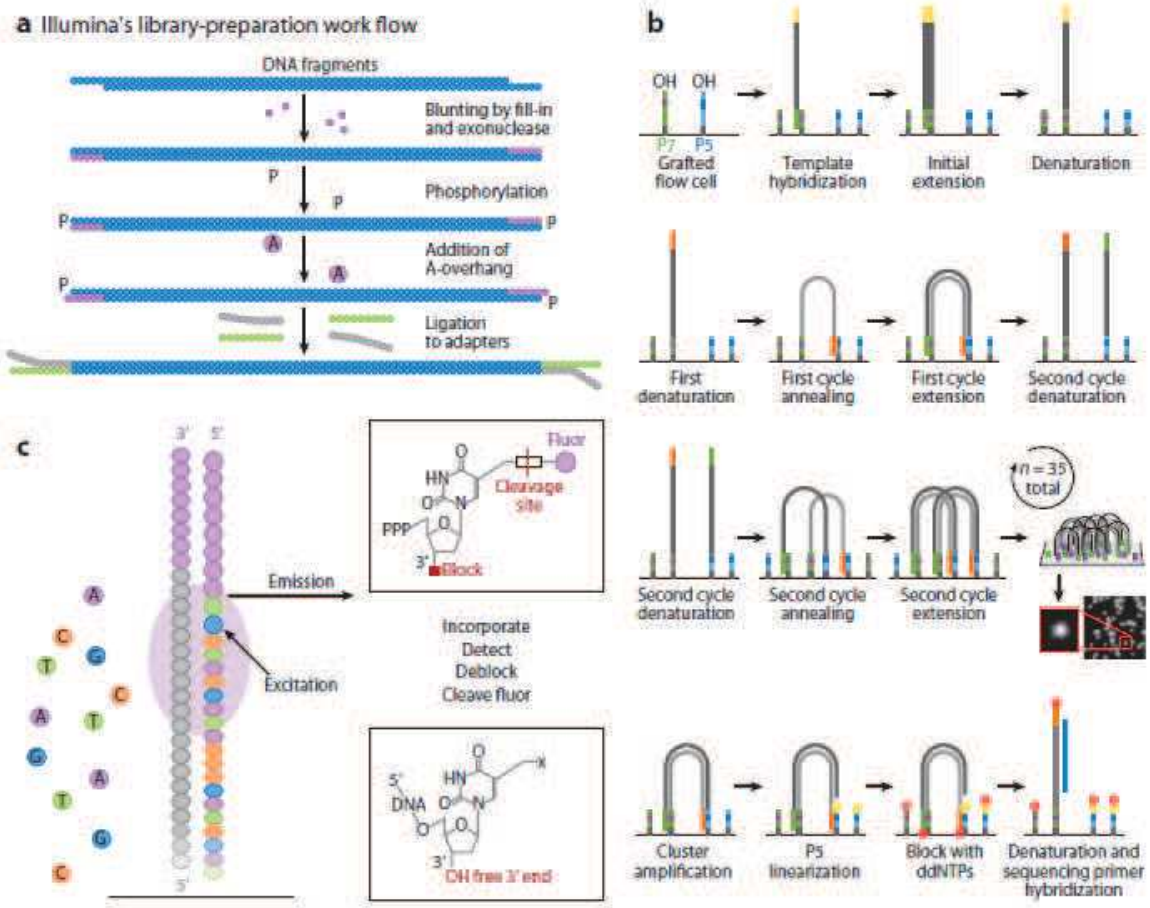

Figure 21 : Principe du séquençage Illumina. D'après Mardis (2013)

A l'heure actuelle, un séquenceur comme le HiSeq 2500, peut séquencer en une seule analyse ou « run » jusqu'à 4 milliards de fragments en « paired-end » (séquençage de deux extrémités de fragments courts), d'une taille maximale de 150 pb. Moins couteux et plus rapide, le dernier né des séquenceurs Illumina, le MiSeq, est un séquenceur de paillasse pouvant produire des « reads » jusqu'à 2 x 300 pb en un seul run. Ainsi, avec un rendement de 25 millions de « reads » par run, le MiSeq permet un large éventail d'applications, du séquençage de gènes ciblés à la métagénomique.

1.4.3. Les technologies de troisième génération

Bien que les performances des méthodes de seconde génération ne cessent de s'améliorer, les techniques de troisième génération ont déjà vu le jour. La différence majeure avec la précédente génération réside sur la capacité à séquencer directement des molécules d'ADN individuellement en évitant l'étape préalable d'amplification et ainsi les biais associés à cette technique (Metzker, 2009). Les séquenceurs de troisième génération sont représentés par les technologies Nanopore (Oxford Nanopore Technologies), HeliScope (Helicos Biosciences) et PacBio RS (Pacific Biosciences). Actuellement la seule technologie de 3ème génération réellement utilisée est le PacBio RS basé sur une technique de séquençage en temps réel d'une seule molécule (SMRT : Single Molecule Real Time sequencing) (Eid *et al*., 2009). Avec une longueur moyenne de « reads » de plus de 1400 pb, une précision de plus de 99%, une préparation de l'échantillon d'environ 30 minutes et des données produites en quelques minutes seulement, le nouveau PacBio RS II, apparaît comme l'une des technologies les plus prometteuses des années à venir (Mosher *et al*., 2014).

1.4.4. Le traitement des données

1.4.4.1. Données issues de l'ADN

L'avènement des méthodes de séquençage à haut débit a engendré des projets de séquençage de grande envergure, tel que le « Global Ocean Sampling » (Venter *et al*., 2004; Rusch *et al.,* 2007; Yooseph *et al*., 2007) ou TARA Oceans (Karsenti *et al*., 2011), générant ainsi des quantités massives de données. De telles études ont ainsi généré un besoin imminent d'outils bioinformatiques pour le traitement de ces données. De nombreuses plateformes d'analyse de données ont vu le jour comme MOTHUR (Schloss *et al*., 2009), QIIME (Caporaso *et al.,* 2010) ou UPARSE (Edgar, 2013). Les principales étapes consistent à filtrer les « reads » bruts en fonction de leur qualité et de les grouper en unités taxonomiques (OTUs) en se basant sur les similarités de séquences. Les séquences représentatives de chaque OTUs sont ensuite assignées en utilisant des bases de données de référence et alignées de façon à créer un arbre phylogénétique. Afin de caractériser la diversité au sein d'un

échantillon, on peut distinguer (i) les indices de diversité alpha, qui permettent de quantifier l'hétérogénéité de la biodiversité au sein de l'échantillon, comme par exemple les indices de diversité de Shannon ou de Simpson, (ii) les estimateurs de richesse spécifique tels que le Chao ou encore l'estimateur ACE et (iii) les courbes de raréfaction utilisées pour valider l'effort de séquençage. La mesure de la diversité bêta est quant à elle utilisée pour comparer la diversité des espèces entre les différents échantillons étudiés (Figure 22).

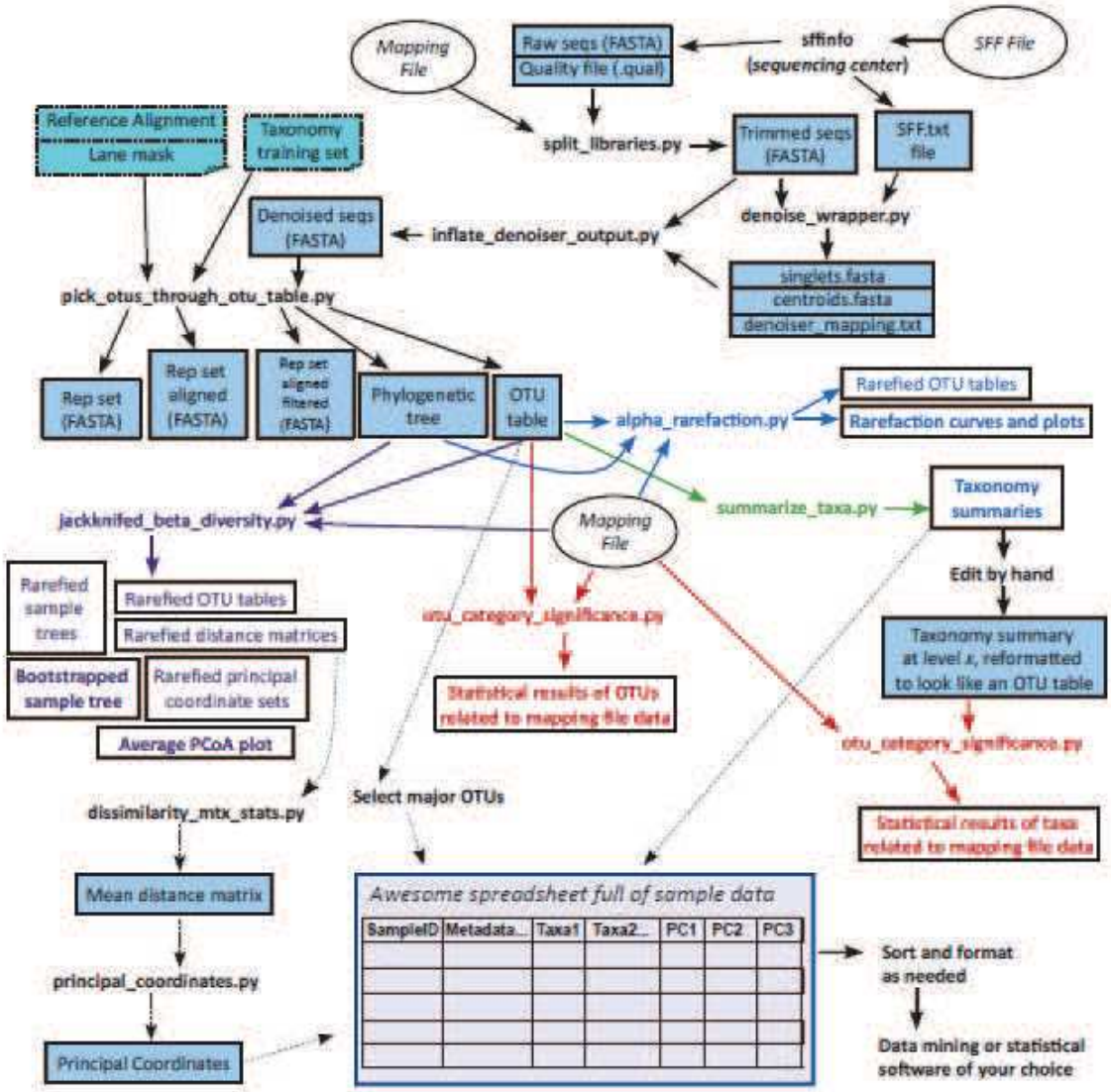

Figure 22 : Etapes caractéristiques pour l'analyse de données 454.

(http://www.wernerlab.org/)

1.4.4.2. Données issues de l'ARNm

Le séquençage massif d'ADN complémentaire (RNAseq) offre la possibilité de quantifier l'expression des gènes, donnant ainsi accès à un premier aperçu des fonctions utilisées dans une communauté. Le séquençage direct des ARN rétrotranscrits permet de déterminer, indépendamment de la composition taxonomique de la communauté étudiée, l'information fonctionnelle. Deux stratégies sont habituellement utilisées pour la reconstruction du transcriptome, (i) l'alignement par rapport à un génome de référence ou (ii) un assemblage *de novo* du transcriptome. Trinity est à l'heure actuelle, le logiciel le plus efficace pour obtenir des transcrits complets dans un large éventail de niveaux d'expression et avec une sensibilité similaire aux méthodes qui s'appuient sur des alignements de génomes. Trinity permet la reconstruction des transcriptomes de tout type d'échantillon, en particulier en l'absence de génome de référence (Grabherr *et al*., 2011). Basée sur des similarités entre séquences déjà annotées, l'annotation fonctionnelle permet d'inférer une fonction à une séquence. A titre d'exemple, Blast2Go est un outil informatique qui permet l'annotation fonctionnelle, structurée en trois catégories : les fonctions moléculaires, les processus biologiques et les composants cellulaires (Conesa *et al*., 2005).

1.5. Objectifs de l'étude

Depuis une vingtaine d'année, les environnements extrêmes ont révélés de nombreuses espèces archéennes et bactériennes aux caractéristiques morphologiques, métaboliques et physiologiques originales. Cependant les micro-eucaryotes et plus particulièrement les champignons n'ont été que très peu étudiés dans ces écosystèmes singuliers. Dans ce contexte, l'objectif de ce travail de thèse était de mettre en évidence et de caractériser par approche moléculaire et culturale la diversité fongique, au sein de sédiments marins. Peu après le début de cette thèse en Octobre 2011, les communautés fongiques ont été décrites comme dominantes parmi les micro-eucaryotes dans les sédiments marins indiquant un éventuel rôle clé des champignons dans cet écosystème (Edgcomb *et al.,* 2011). Très récemment, la première analyse métatranscriptomique de la biosphère profonde (Orsi *et al.*,

2013b) a montré que la composante fongique reste active au sein de la biosphère de subsurface, représentant de 3 à 20% des transcrits.

Bien que les connaissances de la composante fongique dans cet écosystème commencent à se développer, de nombreuses interrogations restent actuellement sans réponses. Dans le cadre de cette thèse cinq questions majeures ont été abordées :

- ✓ Quelle est l'abondance et la composition des communautés microbiennes de subsurface jusqu'à près de 2km sous la surface du plancher océanique ?
- ✓ Parmi les communautés eucaryotes, quelle est la proportion de champignons, et sont-ils actifs ?
- ✓ Peut-on cultiver les champignons endémiques des sédiments marins profonds, et jusqu'à quelle profondeur ? Comment s'adaptent-ils à l'environnement sédimentaire ?
- ✓ Quel est le rôle écologique des communautés fongiques au sein de la biosphère profonde ?

Chapitre II : RESULTATS

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Ce travail de thèse s'est concentré sur les couches sédimentaires très profondes du bassin de Canterbury, au large de la Nouvelle-Zélande (IODP Leg 317). Lors de cette première étude, en étroite collaboration avec le Laboratoire de Microbiologie des Environnements Extrêmes (UMR 6197, UBO), une analyse de l'ensemble des trois domaines du vivant (bactéries, archées et eucaryotes) a été menée afin de révéler la diversité et l'abondance de ces communautés dans cette matrice sédimentaire. En effet, les études s'intéressant conjointement aux diversités procaryotes et eucaryotes dans les sédiments marins de subsurface n'avaient jamais été menées dans les couches profondes et aujourd'hui encore, nous ne savons toujours pas à quelle profondeur se trouve la limite inférieure de la biosphère souterraine profonde.

A travers ce travail, différentes approches ont été utilisées : séquençage haut-débit sur amplicons (pyroséquençage 454, Roche) ciblant l'ARNr 16S et 18S, comptages cellulaires, quantification par PCR en temps réel (marqueurs génétiques et fonctionnels) ainsi que des cultures. Ma contribution dans ce travail collaboratif a été à la fois d'analyser les données de diversité eucaryote mais aussi de mettre en place des cultures d'enrichissement afin de mettre en évidence dans ces échantillons des cellules eucaryotes par hybridation *in situ*.

Nous avons ainsi montré que les procaryotes et les eucaryotes sont présents jusqu'à des profondeurs records malgré la combinaison de contraintes physiques et chimiques inhérentes à ce type d'écosystème. Les limites de profondeurs déjà connues ont ainsi été

repoussées de 518 à 1922 mbsf pour les bactéries et de 159 à 1740 mbsf pour les eucaryotes. Comme attendu, la profondeur a été désignée comme étant le facteur principal expliquant la distribution microbienne et la composition des communautés. Ces résultats nous ont permis de procéder à une analyse la plus exhaustive possible de la diversité microbienne présente dans ces écosystèmes et également de discuter des limites physico-chimiques de la vie. Parmi les eucaryotes, quelques séquences de protistes ont été détectées jusqu'à 583 mbsf, mais la plus grande majorité des séquences appartiennent à des lignées fongiques (entre 56 et 100% des séquences selon la profondeur) affiliées aux phyla des ascomycètes et des basidiomycètes qui, bien qu'ubiquistes, avaient auparavant été mises en évidence dans les sédiments marins ou dans des environnements extrêmes.

Cette première étude nous a permis de mettre en évidence la part majoritaire des champignons, parmi les microeucaryotes, dans les écosystèmes sédimentaires qui pourraient donc avoir un impact significatif dans les procédés biogéochimiques de subsurface.

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ORIGINAL ARTICLE

Microorganisms persist at record depths in the subseafloor of the Canterbury Basin

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The subsurface realm is colonized by microbial communities to depths of >1000 meters below the seafloor (m.b.sf.), but little is known about overall diversity and microbial distribution patterns at the most profound depths. Here we show that not only *Bacteria* and *Archaea* but also *Eukarya* occur at record depths in the subseafloor of the Canterbury Basin. Shifts in microbial community composition along a core of nearly 2 km reflect vertical taxa zonation influenced by sediment depth. Representatives of some microbial taxa were also cultivated using methods mimicking *in situ* conditions. These results suggest that diverse microorganisms persist down to 1922 m.b.sf. in the seafloor of the Canterbury Basin and extend the previously known depth limits of microbial evidence (i) from 159 to 1740 m.b.sf. for *Eukarya* and (ii) from 518 to 1922 m.b.sf. for *Bacteria*.
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Keywords: deep biosphere; subsurface life; eukaryote; record depth

Introduction

In addition to terrestrial and marine near-surface habitats, the deep biosphere is considered to be a third realm for microbial life. Subseafloor sediments provide a habitat for large numbers of microbial cells, as revealed by cell counts (Parkes *et al.*, 2000) or CARD-FISH (Schippers *et al.*, 2005). Although recent data have shown that the global biomass in subseafloor sediments is smaller than given by earlier estimates, the deep subseafloor biosphere still constitutes a large fraction (2.9×10^{29} cells) of Earth's living biomass (Kallmeyer *et al.*, 2012).

The subsurface microbiota is diverse and complex, hosting metabolically active communities down to depths of >1000 meters below the seafloor (m.b.sf.), as revealed by molecular, metagenomic and metatranscriptomic studies (Lipp *et al.*, 2008; Roussel *et al.*, 2008; Biddle *et al.*, 2011; Pawlowski *et al.*, 2011; Orsi *et al.*, 2013a). It harbors representatives from the three domains of life, for example, numerous endemic and/or as yet uncultured *Archaea* and *Bacteria* (for example, Inagaki *et al.*, 2006; Orcutt *et al.*, 2011), in addition to bacterial endospores (Lomstein *et al.*, 2012), protists and fungi belonging to *Eukarya* (Schippers and Neretin, 2006; Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a,b). Occurrence of capsid-encoding organisms has also been confirmed (Engelhardt *et al.*, 2011). Although in subsurface sediment shallower than 1000 m.b.sf. background molecular data on bacterial and archaeal lineages exists (for example, Inagaki *et al.*, 2006; Orcutt *et al.*, 2011), most deep-subsurface microorganisms detected so far were refractory to cultivation (Sass and Parkes, 2011). The diversity of deeply buried microorganisms remains untapped, as subseafloor prokaryotic culturability in most studies is <0.1% of all microscopically detected cells (D'Hondt *et al.*, 2004). Remarkably, when wide enrichment collections targeting different physiological groups such as fermenters, sulfate-reducers and methanogens were performed using different subseafloor sediments, these often led to the isolation of the

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same few 'generalist' bacteria (for example, Batzke *et al.*, 2007). In most cases, the retrieved bacterial genera were adapted to a broader spectrum of environmental conditions (for example, broad temperature range for growth) compared with their surface counterparts (Sass and Parkes, 2011).

So far, within subseafloor sediments, active *Bacteria* have been identified down to 518 m.b.sf. (Bale *et al.*, 1997), active *Archaea* down to 1626 m.b.sf. (Roussel *et al.*, 2008) and active microeukaryotes down to 159 m.b.sf. (Orsi *et al.*, 2013a), but we are still eager to know the depth limit of the deep subsurface biosphere. Limits to microbial habitability in subseafloor sediments are set by a variety of physical and chemical parameters like temperature, pH, pressure, salinity, porosity, availability of energy, nutrients and water and maybe also by age as there was water exchange within the sediment pores. The present study site is not characterized by particularly extreme conditions but stands out from sites previously examined by its depth and low porosity. The depth limit of the deep biosphere remains an important issue to place bounds on the volume of the subseafloor biosphere and to guide the search for deep life capabilities/adaptation and the role of microorganisms in global nutrient cycles. We hypothesized that life could exist in even deeper sediments if pore space was sufficient.

In this study, we investigated the subsurface microbial communities from a core of 1927 m length collected in the Canterbury Basin (344 m water depth), off the coast of New Zealand at site U1352, which was drilled during the Integrated Ocean Drilling Program (IODP) Expedition 317 with DS *Joides Resolution.* Our purpose was to investigate vertical distribution of microbial communities, abundance and evenness of taxa above and below 1000 m.b.sf. depth. We developed a highly stringent massive parallel tagged-amplicon sequencing of 16S–18S hypervariable regions of small-subunit (SSU) rRNA gene (Supplementary Figures S1 and S2; Supplementary Tables S1–S3), coupled with cell counts, real-time PCR (phylogenetic and functional genes) and cultivation approaches. This rigorous method was applied to sediment/carbonate rocks spanning epochs from the Holocene to late Eocene.

Materials and methods

Site description and sampling

Three holes (A, B and C) were drilled at Site U1352 (44°56′26.62″S; 172°1′36.30″E), reaching a total depth of 1927.5 m CSF-A, and thus spanning the Holocene to late Eocene epochs. Fluorescent microspheres were used as tracers for contamination during drilling. Sampling was processed under strict contamination controls onboard and offshore, and only samples with no detectable contamination were used for this study (Fulthorpe *et al.*, 2011). Onboard, only the center parts of unconsolidated sediments and intact pieces of rocks that had been exposed to ultraviolet radiation after washing were kept for microbiological analyses, as reported elsewhere (Expedition 317 Scientists, 2011). Subsamples were immediately frozen at −80 °C for onshore molecular analyses, stored at 4 °C under an anaerobic gas phase for later cultivation and stored at 4 °C in a 3% NaCl/3% formalin solution for cell counting. Detailed information on sampling/subsampling of sediment, on contamination controls and on depth scale terminology are provided in Supplementary Text.

Lithological, physical and geochemical data

Environmental data were acquired onboard during IODP Expedition 317, as reported elsewhere (Fulthorpe *et al.*, 2011).

DNA extraction, PCR amplification and contamination controls

DNA extractions were made from 16 samples collected all along the core. In order to avoid contamination, all handling was carried out in a PCR cabinet exclusively dedicated to low biomass sediment samples (PCR cabinet; CaptairBio, Erlab, Val de Reuil, France), using Biopur 1.5 ml Safe-Lock micro test tubes (Eppendorf, Le Pecq, France), ultrapure PCR water (Ozyme, St Quentin en Yvelines, France) and ultraviolet-treated (>40 min) plasticware and micropipettes. Negative controls (reaction mixture without DNA) were included in each set of PCR reactions. In addition, a negative control (for example, negative DNA extraction) was prepared for each work stage, to ensure that no contamination with exogenous amplifiable DNA occurred during the different stages of sample treatment. The FastDNA Spin Kit for Soil (No. 6560-200, MP Biomedicals, Strasbourg, France) was used to perform DNA extractions, with few modifications. Detailed information on DNA extractions and PCR amplifications are provided in Supplementary Information. Primer sequences used in this study are detailed in Supplementary Table S2, and primer sets for direct and nested PCR amplifications are detailed in Supplementary Figure S2.

454-Pyrosequencing

For each DNA extract, four independent 25-μl PCR amplifications were run with fusion primer pairs specific for *Bacteria*, *Archaea* and *Eukarya*, as detailed in Supplementary Table S3. PCR products were pooled two by two, so as to have two independent replicates for pyrosequencing. Potential contaminants from lab reagents were excluded through the sequencing of negative-control samples and the removal of operational taxonomic units (OTUs) containing sequences retrieved in negative controls. Detailed information on 454-pyrosequencing, quality filtration,

trimming, clustering and taxonomic affiliation are provided in Supplementary Text.

Cell counts
Total prokaryotic cells were enumerated in triplicate from 13 uncontaminated sediment samples collected all along the core, using the cell extraction protocol (protocol FCM-A) described by (Morono *et al.*, 2011) until step 9. Then, all supernatants containing extracted cells were filtered onto 0.2-μm filters (Anodisc, Whatman, Versailles, France) and stained with SYBRGreen I (Invitrogen, Cergy Pontoise, France), as described elsewhere (Noble and Fuhrman, 1998). Filters were counted in epifluorescence mode, with an Olympus BX60 microscope (Rungis, France) (objective ×100, pH3, WIB filter; details in Supplementary Text).

Real-time PCR measurements
Quantifications of different lineages and diverse functional genes were performed all along the core by quantitative, real-time PCR. Quantifications of *Bacteria*, *Archaea*, *Eukarya*, JS1-*Chloroflexi* and *Geobacteriaceae* were performed using previously described quantitative PCR assays based on the detection of 16S or 18S rRNA (Schippers *et al.*, 2012). These assays were carried out using the *Taq*Man or SYBRGreen chemistries. DNA copy numbers were also determined for the following functional genes: *mcrA* for alpha subunit of the methyl coenzyme M reductase, *dsrA* for the alpha subunit of the sulfite (bi)reductase, *aprA* for the alpha subunit of the adenosine-5′-phosphosulfate reductase and *cbbL* for the large subunit of the enzyme ribulose-1.5-bisphosphate carboxylase/oxygenase (RubisCO, form I 'red-like'), as described elsewhere (Schippers *et al.*, 2012).

Cultures and approaches used for their analysis
A sediment slurry membrane system was used for cultivation (Ferrari *et al.*, 2008) (Supplementary Figure S8; details in Supplementary Information). Different anaerobic metabolisms found in the subsurface biosphere were targeted in culture: fermentation, sulfate reduction and methanogenesis/acetogenesis. Media, culture conditions, viability and identification procedures of cells are described in Supplementary Text.

Statistical analyses
Principal component analysis was used to help in visualization of high-dimensional data. An order abundance matrix was combined with environmental parameters, using XLSTAT, to assess relationships between microbial taxa and ecological variables (Addinsoft USA, New York, NY, USA). A second complementary approach was based on regularized canonical correlation analyses, which were performed to highlight correlations between the order abundance matrices (X) and the environmental parameters (Y) using the R software CCA package.

Results and discussion

Core description
The core lithology was characterized by horizontal gradual layers, from unconsolidated sediments (clay, marl) to carbonate rocks (Figure 1). The core was composed of three lithological units (UI, UII and UIII). Unit I (0–711 m CSF-A, meters of core depth below seafloor computed by conventional method A, corresponding to m.b.sf. (see 'IODP depth scale terminology' at www.iodp.org/program-policies/) was predominantly characterized by a transition from mud-rich sediment to marl. Unit II (711–1853 m CSF-A) consisted of hemipelagic/pelagic sediment from calcareous sandy mud to sandy sandstone. Unit III (1853–1924 m CSF-A) was characterized by a sharp change (Marshall unconformity: ~12 Ma are missing) that occurred at 1853 m CSF-A and was formed of hemipelagic to pelagic foraminifer-bearing nannofossil limestone of early Oligocene to late Eocene age (Figure 1). The temperature at the bottom of the hole was estimated to be in the range of 60 °C–100 °C on the basis of thermal conductivity measurements and geochemical results (Fulthorpe *et al.*, 2011). Below 1000 m CSF-A, sediments were replaced by consolidated sedimentary calcium carbonate rocks with porous horizons of glauconite. Porosity decreased with depth and mean pore size was around 2–4 μm at the hole bottom. In carbonate rocks, numerous fractures and stylolites were observed (Supplementary Figure S3). Organic carbon content was low (<0.6 wt %), with only a few samples having >1 wt % total organic carbon (Figure 1). The organic matter quality changed from relatively labile volatile material in the shallower sediments to more stable protokerogen with increasing depth. Methane and ethane both occurred below 11.7 and 18.2 m CSF-A, and the relative ethane content increased with increasing burial depth and temperature (Figure 1). Low but increasing concentrations of C3-C5 and occasionally C6 alkanes were also measured with depth. pH values were close to 7.5 and stable from the surface to 1164 m CSF-A. Sulfate concentration decreased gradually in the first meters of the core and reached the detection limit at ~16 m CSF-A (the SMTZ (sulfate–methane transition zone), was placed between 15.2 and 16.6 m CSF-A), then it remained close to the detection limit (~0.85 mM) down to 1433 m CSF-A (Figure 1).

Vertical distribution of cells
We analyzed and compared cell abundances and cell concentrations reported for different geographic sites using a standardized procedure based on cell extraction and dissolution of silicates (Noble and Fuhrman,

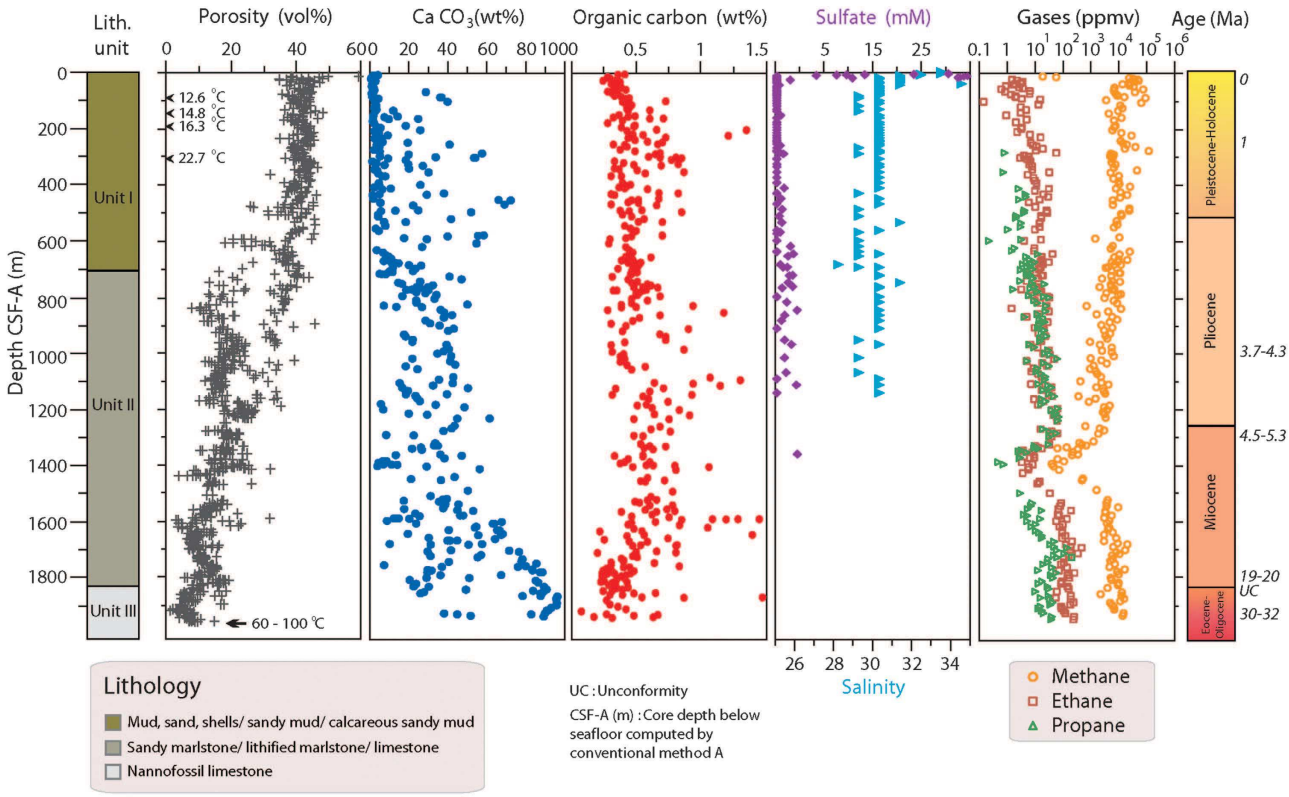

Figure 1 Main physical–chemical characteristics of the studied core. Black arrows indicate measures of *in situ* temperatures used to calculate a thermal gradient of 46 °C km^{-1}. This thermal gradient, together with the interpretation of the thermal maturity gradient defined by T_{max} measurements, allowed estimating a bottom-hole temperature comprised between 60 °C and 100 °C (Source: IODP report 317) (Fulthorpe *et al.*, 2011).

1998; Kallmeyer *et al.*, 2008; Morono *et al.*, 2011) (Figure 2). Mean cell numbers decreased with depth from about $1.5 \times 10^6 \pm 4.7 \times 10^4$ cells cm^{-3} ($n = 8$) at the surface (3.76 and 15.1 m CSF-A) to $2.5 \times 10^4 \pm 4.9 \times 10^3$ cells cm^{-3} ($n = 7$) within the deepest samples (1911 and 1922 m CSF-A). The detection limit, calculated in our conditions (Kallmeyer *et al.*, 2008), was 2.94×10^3 cells cm^{-3}. The depth profile (down to 600 m CSF-A) was consistent with the general depth distribution of prokaryotic cells from other subsurface sediments (Kallmeyer *et al.*, 2012).

Vertical distribution of microbial taxa

It is not clear what controls abundance of *Bacteria* and *Archaea* within deep marine sediments (Schippers *et al.*, 2005; Lipp *et al.*, 2008; Schippers *et al.*, 2012). Here, a real-time PCR approach was applied to quantify representatives of the three life domains. Calculated detection limits for *Bacteria*, *Archaea* and *Eukarya* were respectively 1.6×10^4, 1.1×10^3 and 2.9×10^3 SSU rRNA gene copies per gram of sediment (wet weight). *Archaea* were the most abundant within the first meters, while *Bacteria* dominated the rest of the core (Figure 2). Archaeal SSU rRNA gene copy numbers strongly decreased with depth (from 1.8×10^6 to 1×10^3 gene copies g^{-1}, corresponding roughly to 1×10^6 to 6×10^2 cells g^{-1}) and were no longer detectable below 650 m CSF-A. A similar depth distribution was observed for eukaryotic SSU rRNA gene copy numbers, but abundances were relatively constant with depth ($\sim 10^4$ copies g^{-1}). Bacterial SSU rRNA gene copy numbers were low ($\sim 10^6$ copies g^{-1} $\approx 2.5 \times 10^5$ cells g^{-1}) at the surface and decreased with depth up to 1600 m CSF-A (8×10^4 copies g^{-1} $\approx 2 \times 10^4$ cells g^{-1}).

Along with these measures, deep sequencing allowed the detection limits to be lowered and masked lineages to be revealed. We pyrosequenced bacterial (V4-V5), archaeal (V1-V3) and eukaryotic (V1-V3) SSU rRNA gene amplicons from 16 depth horizons and one negative control, pooled together in one single data set with two PCR replicates per sample to overcome PCR and sequencing errors (Supplementary Figure S1). Sequences were grouped into OTUs with a 97% identity threshold. Sequence composition of the OTUs was then analyzed, and OTUs entirely composed of sequences that had appeared in a single PCR only were excluded from the diversity analyses. All the sequences kept appeared at least twice independently. Potential contaminants from laboratory reagents were excluded through the sequencing of negative-control samples and the removal of OTUs containing sequences retrieved in negative controls.

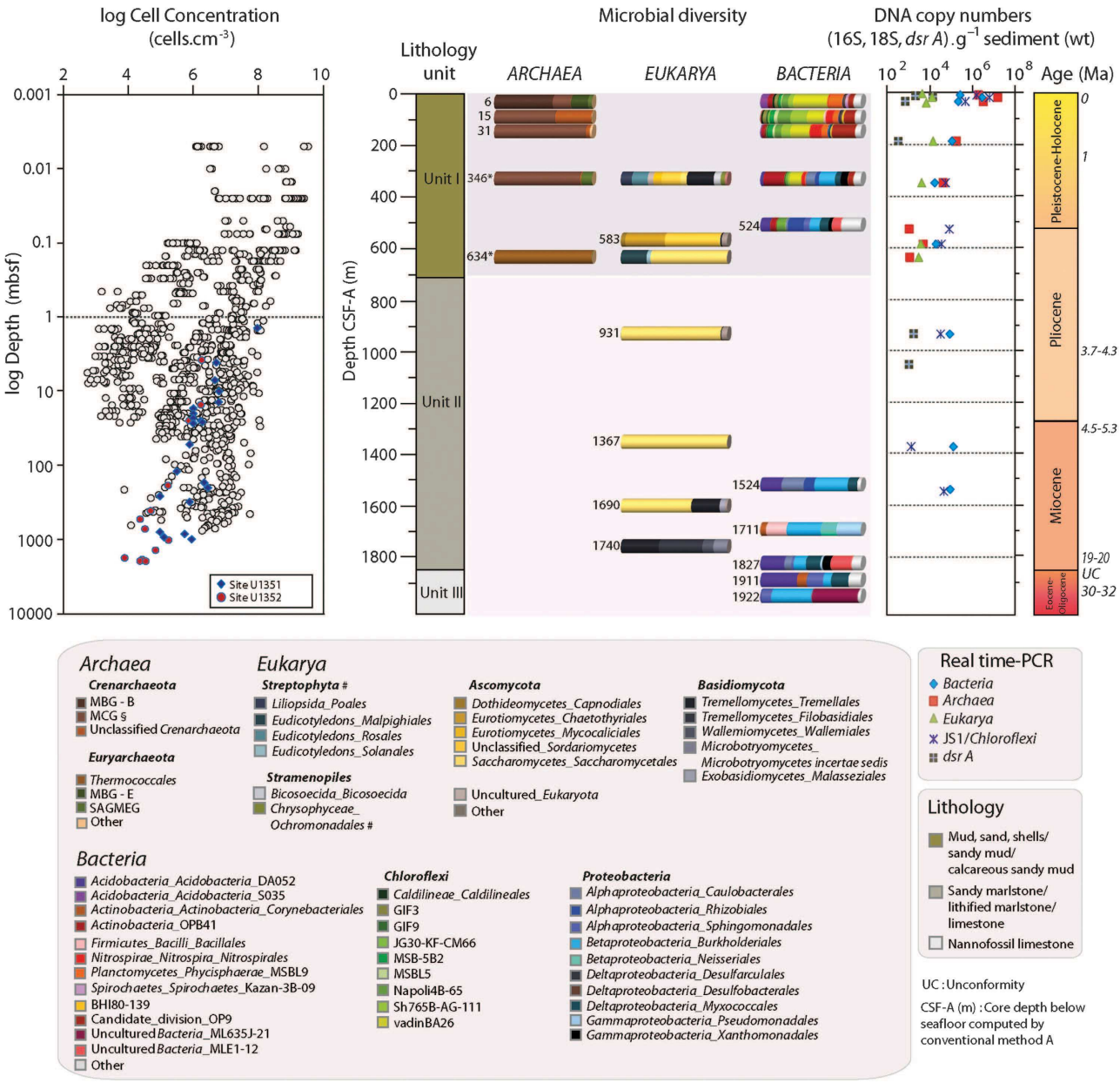

Figure 2 Lithological structure and age of lithological deposits at site U1352 in the Canterbury Basin compared with depth distribution of cell counts, 16S/18S rRNA gene-tag sequences and DNA copy numbers of genetic markers and functional genes. On the left, cell concentrations counted at site U1352 (red dots) and neighboring site U1351 (blue squares), according to depth (m CSF-A), compared with the general depth distribution of cells (grey dots) in subseafloor sediments (Kallmeyer *et al.*, 2012). In the center, Phylum Class_Order distribution of archaeal, eukaryotic and bacterial 16S/18S rRNA gene-tag sequences (based on SILVA111 classification) from OTU containing ⩾100 sequences (the remaining sequences were grouped into 'Other'). On the right, copy numbers of the disulfite reductase genes A, of the 18S rRNA genes from total *Eukarya* and of the 16S rRNA genes from total *Bacteria*, *Archaea* and *JS1/Chloroflexi*-related bacteria. *Geobacteriaceae* were detected only up to 15 m CSF-A ($\sim 5 \times 10^3$ copies per g). No amplification from greater depth was shown. The functional genes *mcrA*, *aprA* and *cbbL* were not detected at all. § In other classifications, MCG affiliate with the *Thaumarchaeota*. #Lineages of plants and algae. *Depth horizons where ⩾100 reads of plants and Chrysophyceae were detected.

The remaining OTUs were used to calculate non-parametric diversity indices (Figure 3, Supplementary Figures S4 and S6) and compared with the SILVA 111 database for taxonomic affiliation. Pyro-sequencing results were congruent with the data discussed above. Archaeal sequences could not be amplified and sequenced for samples <634 m CSF-A, as observed with real-time PCR analyses. The non-detection of archaeal 16S rRNA genes <650 m CSF-A using two different amplification methods suggests that *Archaea* are likely rare or absent at great depths in the Canterbury Basin. Eukaryotic sequences were detected down to 1740 m CSF-A, and bacterial sequences were found up to the maximal depth of 1922 m CSF-A. The observed species richness (that is, number of OTUs) was

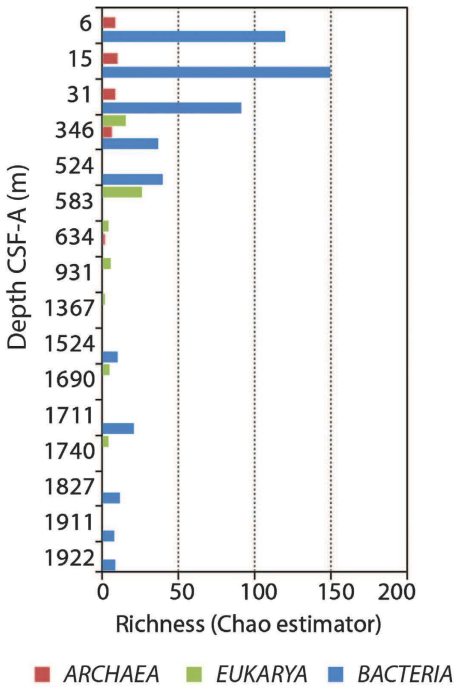

Figure 3 Community richness (Chao1 non-parametric estimator) for archaeal, eukaryotic and bacterial sequences making up the OTUs (calculated by MOTHUR at 3% difference between OTUs).

extremely low in comparison with other microbial habitats investigated so far, including extreme environments (for example, Roalkvam *et al.*, 2012). Indeed, only 198, 16 and 40 unique bacterial, archaeal and eukaryotic OTUs, at 3% dissimilarity level, were detected in the entire cored sequence (Supplementary Figure S4, Supplementary Tables S4 and S5). Chao1 estimator revealed a vertical decrease in microbial richness with increasing depth (Figure 3). Richness estimates for *Archaea* and *Eukarya* dropped off gradually with depth and reached only two and four OTUs, respectively, at the deepest depth for which a PCR signal was obtained. Beta diversity estimators (that is, diversity among samples) revealed a strong differentiation between communities with depth and a strong vertical structuration (Supplementary Figure S5).

Archaeal diversity showed high abundances of MBG-B (Marine Benthic Group B) and MCG (Miscellaneous Crenarchaeotal Group), two archaeal groups typically found in subseafloor sediments (Lloyd *et al.*, 2013). Representatives of the as-yet-uncultured lineages MBG-B, MBG-E (Marine Benthic Group E) and MCG were the predominating taxa in surficial layers, while MCG was the most consistently detected archaeal lineage down to 346 m CSF-A (Figure 2). MBG-B and MCG members are heterotrophic *Archaea* frequently found in surficial marine sediments (Biddle *et al.*, 2006; Lloyd *et al.*, 2013). *Thermococcales* dominated archaeal diversity of the sediment horizon at 634 m CSF-A. Methanogens and anaerobic methanotrophs were not detected, in agreement with the real-time PCR analysis for *mcrA*. Their absence from the data set might be due to the intervals sampled, which do not correspond to the SMTZ.

In *Eukarya*, few protist OTUs (Stramenopiles and uncultured *Eukaryota*) were detected down to 583 m CSF-A. Sequences affiliated with the bacterivorous protists *Bicosoecida* were detected at 346 m CSF-A, raising the question of the existence of a subsurface complex trophic web. In agreement with recently published papers (Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a,b), fungi appeared to be the most frequently detected eukaryotes in the Canterbury Basin, with 56–100% of the SSU rRNA gene sequences. Different shifts between *Ascomycota* and *Basidiomycota* were observed along the core (Figure 2). *Tremellomycetes* (order *Tremellales*), *Sordariomycetes* and *Eurotiomycetes* dominated shallow depths while *Saccharomycetes* were detected at depths between 630 and 1365 m CSF-A. Deeper layers were dominated by *Wallemiomycetes*, *Microbotryomycetes* and *Tremellomycetes* (order *Filobasidiales*, not found at shallow depths). These heterotrophic fungi have been described in deep sediments of other locations (for example, Nagano and Nagahama, 2012; Richards *et al.*, 2012) and demonstrated to be active members of microbial communities (Orsi *et al.*, 2013b). So, fungi represent an important component of sediment ecosystems through their impact on nutrient cycling and mineral weathering.

Bacteria were dominated by *Chloroflexi* and *Proteobacteria*, two heterotrophic bacterial groups well represented in subsurface sediments (Figure 2). They comprised 67% of the sequences and 69% of the OTUs in total. However, the abundances of the two phyla were negatively correlated. *Chloroflexi* dominated microbial communities at shallow depths (>600 m CSF-A), and their abundances and richness decreased rapidly. Reciprocally, *Proteobacteria* were found all along the core, but their relative abundance showed a sharp increase <343 m CSF-A. Among the other lineages observed in this study, *Planctomycetes*, *Nitrospirae* and the candidate division OP9 were major contributors of the amplicon pool at shallow depths. Below 600 m CSF-A, *Acidobacteria*, *Firmicutes* (a phylum containing spore-formers) and two loosely defined groups of uncultured *Bacteria* (ML635J-21 and MLE1-12) were the most consistently detected lineages. Real-time PCR quantification of the JS1-*Chloroflexi* group confirmed these results as $\sim 10^3$–10^6 SSU rRNA gene copies g^{-1} were detected between the sediment surface and 1532 m CSF-A. *Deltaproteobacteria* were detected above the SMTZ and at great depths. Genes encoding a functional dissimilatory sulfite (bi)reductase (*dsrA*), a key enzyme of dissimilatory sulfate reduction frequently encountered among *Deltaproteobacteria*, was quantified above the SMTZ and in layers up to 1000 m deep in the sediment. The gene became undetectable below this depth, either because it may decrease below the detection limit or because the detected *Deltaproteobacteria* cannot respire sulfate.

Diversity and environmental factors
Principal component analyses coupled with regularized canonical correlation analyses were performed to visualize relationships between environmental factors and microbial taxa. We first evaluated the relationships between all environmental parameters measured (that is, depth, pH, salinity, porosity, alkalinity and concentrations of calcium, calcium carbonate, ammonium, magnesium, sulfate, inorganic carbon, organic carbon, methane and ethane) to design a network of correlations. Only the six most explanatory variables were kept (Supplementary Figure S6). This complementary analysis reinforced the conclusion about microbial distribution pattern and vertical community composition, depth being defined as a main factor explaining diversity changes (Supplementary Figure S7).

Handling deeply buried microorganisms
Cultivation approaches allowed prokaryotic and eukaryotic strains to be grown, corresponding to a fraction of the microbial communities detected all along the core, underlining that these microorganisms were viable. Fungal strains were obtained at 21–765 m CSF-A, using elevated hydrostatic pressure to mimic *in situ* conditions (Figures 4a–c, Supplementary Table S6). Sequencing of the ITS1 rRNA regions allowed identification of a *Cadophora* representative that had already been found in extreme environments, that is, Antarctic environments (Tosi *et al.*, 2002) and deep-sea hydrothermal vents (Burgaud *et al.*, 2009) (Supplementary Table S6). Fifty-seven anaerobic fungi, currently under description, have also been isolated from these sediments (Rédou and Burgaud, unpublished data). In addition to the important finding that living fungi could be cultivated from the sediment samples, microbial colonies were grown anaerobically at 60–70 °C from calcareous chalk/limestone samples collected at 1827 and 1922 m CSF-A (Figures 4d and e), using a microcultivation method (Supplementary Figure S8). The microcolonies were successfully transferred to liquid media and subcultured. From the different tests performed, it was impossible to grow true methanogens and true sulfate-reducers. Only bacterial fermentative strains degrading the organic compounds supplied (that is, low quantity of yeast extract) have grown. Within these subcultures, mean cell densities were low, around 4×10^5 cells ml^{-1} and growth rates were slow (in 2.5 years of culture, only 6–9 subcultures at 1/40 or 1/50 have been

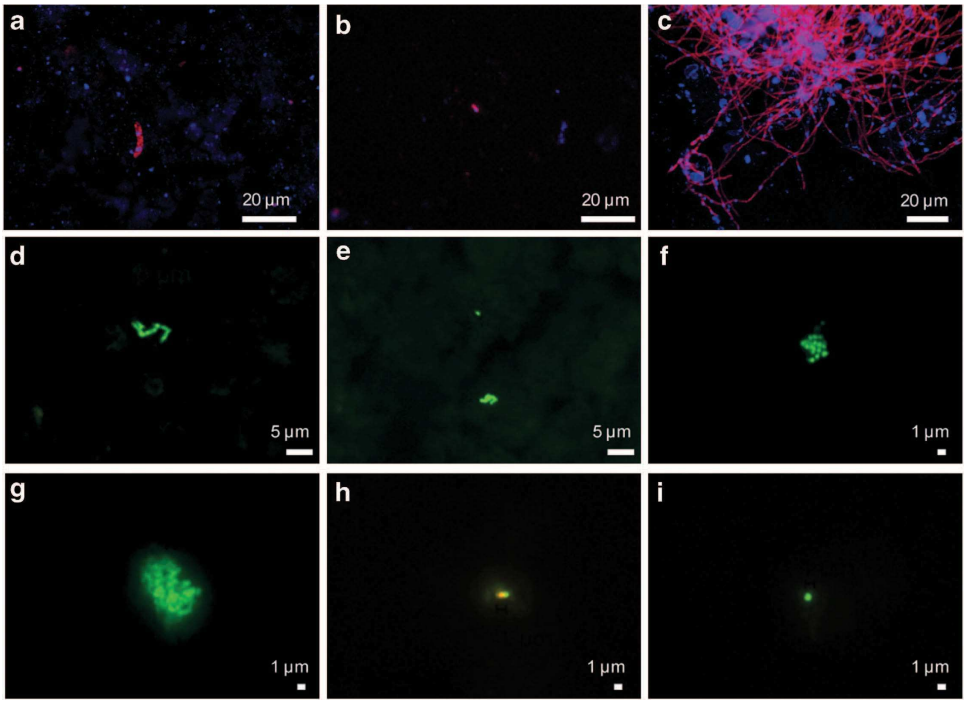

Figure 4 Epifluorescence microphotographs of initial enrichment cultures and subsequent liquid subcultures hybridized with the Cy3-labelled probe Euk516 (staining of nuclei with DAPI) (top panel) or stained with SYBRGreen I (middle panel + **g**) or with the dual staining LIVE/DEADBacterial Viability Kit (**h** and **i**). (**a–c**) Identification of fungus-like eukaryotic cells with fluorescently labeled 18S rRNA oligonucleotide probes in initial enrichment cultures with sediments from 21 m CSF-A on PDB 3% at 4 MPa (**a**), with sediments from 37 m CSF-A on PDB 3% at 11 MPa (**b**) and with sediments from 765 m CSF-A on PDB 0% at 11 MPa (**c**). (**d**, **e**) Microcolonies observed on polycarbonate membranes (initial enrichment cultures) after 15 days of incubation with sediments from 1922 m CSF-A on H_2/CO_2 + YE. (**f**, **g**) Cellular aggregates observed in the seventh liquid subcultures performed after the initial enrichment with sediments from 1827 m CSF-A on YE + peptone + casamino acids. (**h**, **i**) Cell structural integrity was observed in the seventh liquid subcultures performed after the initial enrichment with sediments from 1827 m CSF-A on acetate + YE (**h**) and with sediments from 1922 m CSF-A on YE + peptone + casamino acids (**i**).YE, yeast extract; PDB, potato dextrose broth.

performed). Cells were able to grow at atmospheric pressure and at the estimated *in situ* pressure (22 MPa). They were composed of viable very small rods, coccobacilli and cocci of 300–800 nm in diameter, often forming aggregates (Figures 4f–i). These small sizes and this cellular organization as consortia raises questions about the living conditions of these cells and their (in)dependence with regard to other cells. The smallest diameter of a cell that assures its viability was calculated as ~200 nm (Velimorov, 2001). The major lineages identified in DNA and RNA libraries from these subcultures belonged to *Alpha*-, *Beta*-, *Gamma-proteobacteria*, *Actinobacteria* and *Armatimonadetes* (Figures 5). With the exception of *Armatimonadetes*, all these taxa were detected from pyrosequencing in crude samples from 1827 to 1922 m CSF-A. The majority of the sequences had relatives recovered from environments with similar physical–chemical characteristics (Lin *et al.*, 2006; Mason *et al.*, 2010; that is, hot and reduced habitats) compared with the Canterbury subseafloor. Considering the 'ubiquity' of these taxa, one can hypothesize that they are generalist bacteria, which would have been

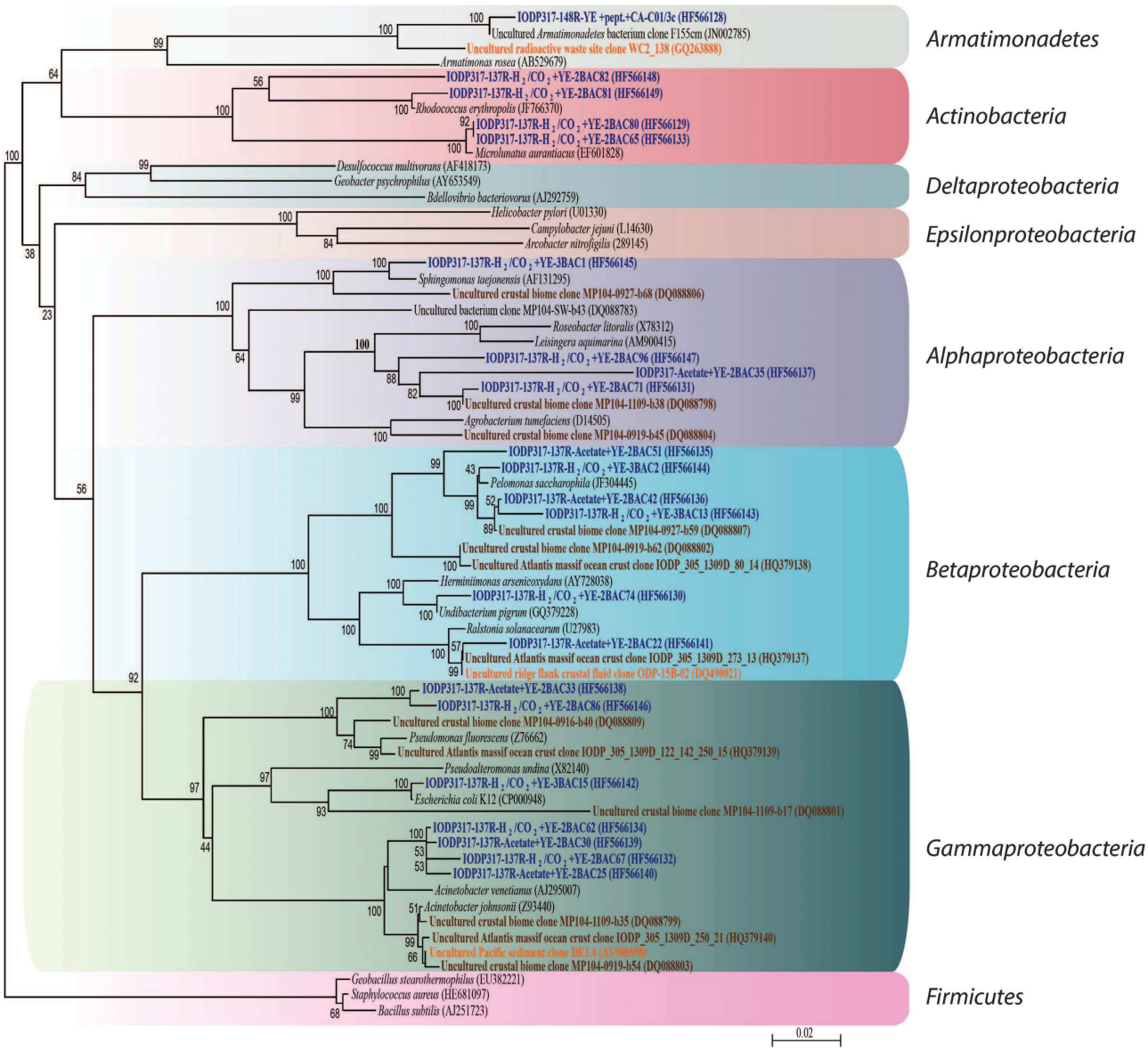

Figure 5 Phylogenetic position of the bacterial 16S rRNA gene sequences detected in RNA extracts from the fifth to eighth liquid subculture following the initial enrichment step on a sediment substrate membrane system. The phylogenetic reconstruction was performed using the Neighbor-Joining algorithm. Bootstrap values based on 1000 replications are indicated at the branch nodes. Representative bacterial 16S rRNA gene sequences of H_2/CO_2 + yeast extract (YE)-based cultures and acetate + YE-based cultures from the 137R section (1827 m CSF-A), as well as YE + peptone (pept.) + casamino acids (CA)-based culture from the 148R section (1922 m CSF-A) are shown in bold blue letters. Sequences from basalt or an underground aquifer where the temperature is high ($52 < T < 80$ °C) are shown in brown; sequences from environments rich in hydrocarbons or radioactive metals are shown in orange. Accession numbers are given in brackets.

maintained during progressive burial of sediments or by transportation through circulating fluids. They might have acquired metabolic capabilities enabling them to resist the associated environmental changes. However, this hypothesis needs to be analyzed in detail. Furthermore, similar SSU rRNA gene sequences do not automatically correspond to identical physiologies, identical phenotypes or similar functions.

Impact of potential contaminants on native microbial populations

Contamination is a crucial issue when working with subseafloor sediments. In general, contamination during drilling is still difficult to predict. During IODP Expedition 317, the level of contamination during drilling was evaluated by using fluorescent microspheres, and only samples with no detectable contamination were kept for microbiological analyses. Nevertheless, samples without microspheres are not necessarily uncontaminated (Smith *et al.*, 2000). Contamination generally decreases from the exterior to the interior of both sediment and rocks cores (for example, Lever *et al.*, 2006). In consequence, only the interior of sediment cores and intact pieces of rocks that had been exposed to ultraviolet light after washing were used for the analyses. In addition, for molecular experiments deeply frozen samples of >1 cm in diameter were sterilized by flaming. Afterwards, all possible contaminations during the wet-lab steps have been strictly controlled and minimized (see Supplementary Text). The cutting-edge strategy applied for the pyrosequencing and bioinformatic analyses allowed removing potential spurious sequences and OTUs likely to contain contaminants by sequencing of negative controls, a duplicate procedure and an associated bioinformatics pipeline. In addition to these precautions, the level of potential contamination of our samples was estimated by calculating the number of contaminating cells per gram of sediment and per gram of sedimentary rock based on the mean contamination values with drilling fluids and mean cell abundances in surface waters reported in the literature. The mean potential contamination was estimated as (i) 0.011 ± 0.018 μl of drilling fluid per gram for unconsolidated sediments drilled using advanced piston coring (APC) and (ii) 0.027 ± 0.029 μl g^{-1} for rocks collected using rotary core barrel (Lever *et al.*, 2006). Considering these levels of contamination, mean cell counts of 5×10^5 cells ml^{-1} in surface waters in the ocean (Whitman *et al.*, 1998) and average densities of 1.85 g cm^{-3} in sediments and 1.99 g cm^{-3} in sedimentary rocks at site U1352, potential contamination of the interior of the core sample should be expected very low with 5–11 cells g^{-1} of sediment only. A second reported estimate indicates that <50 cells g^{-1} of sediment contaminated APC core centers drilled with *Joides Resolution* and that XCB cores were generally more contaminated with contamination levels 3–10 times higher in XCB cores than in APC core centers (House *et al.*, 2003). Considering these different estimates of potential contamination, the observed cell counts at site U1352 were 2–5 orders of magnitude higher in the studied samples. If contamination cannot be excluded, in the worst case, non-indigenous cells represent only up to 1% of total cells in the sample. Therefore, it is most likely that $>99\%$ of the counted cells are native to the sampled sediment/rocks. This implies that the vast majority of the prokaryotic and eukaryotic DNA subjected to pyrosequencing was therefore derived from the sediment native cells. By extension, assuming that most of the prokaryotic DNA extracted from sediment samples is from native cells, the fact that cultivated bacteria match OTUs abundant in the crude sediment samples supports the idea that these cultivated strains are isolates of native bacteria. Consequently, the potential impact of contaminants on each category of data (cell counts, molecular data and cultures) is likely very low.

Ecological implications and future prospects

We have underlined that the subseafloor of the Canterbury basin hosts microorganisms that comprise *Bacteria*, *Archaea* and *Eukarya.* Some of these microorganisms are alive and, at least to a certain extent, revivable. The communities exhibit a quite low phylogenetic diversity, but this does not necessarily correspond to a low functional diversity. This poor diversity could be explained if natural selection has produced (i) taxa adapted to harsh subsurface conditions (that is, specialists), which would be expected in the case of a low connectivity among habitats; and/or (ii) taxa with a broad physiological plasticity, allowing them to survive in a diversity of nutritional and physical–chemical conditions (that is, generalists). In fact, some taxa detected through their 16S/18S rRNA gene sequences are thought to be endemic to subsurface habitats, while others seem ubiquitous and are consistently encountered in common and extreme environments. The bacterial strains in cultures are related to opportunistic or generalist taxa isolated from a broad array of redox environments, which raises the question of the existence of microbial metabolic versatility and also questions the species concept, as behind a given name or a given OTU can lay a variety of microorganisms with different ecological lifestyles. Metabolic versatility has already been demonstrated in well-known taxa. For example, some *Thermococcales* strains, which are usually fermenters that reduce sulfur compounds, can grow in oligotrophic conditions or can oxidize carbon monoxide (Sokolova *et al.*, 2004). Heterotrophy is likely to be the major mode of carbon assimilation within microbial communities of subsurface marine sediments (Batzke *et al.*, 2007). Our culture data support this hypothesis. Genome

and metagenome analyses would allow functions to be predicted on a finer scale to assess and hypothesize the individual ecological functions within the analyzed habitat or ecosystem (Vandenkoornhuyse *et al.*, 2010). The detection of fungal sequences at great depths and our success in the cultivation of fungal strains leads us to ask what role they play in deep carbon cycling and what involvement they have in dynamics/regulation of prokaryotic populations, if they are active *in situ*.

The broad polyphasic approach developed in this study provides direct evidence that viable microorganisms can be present in rocks that are hardened but not totally cemented, where stylolites and microfluid circulations exist. Our data demonstrate that the combination of physical, chemical and energetic constraints encountered from 0–1922 m CSF-A in the subseafloor of the Canterbury Basin still allow microorganisms to persist down to at least 650, 1740 and 1922 m CSF-A for *Archaea*, fungi and *Bacteria*, respectively. It extends the subseafloor sedimentary depths at which subseafloor organisms are known to be present to 1740 m for fungi and to 1922 m for *Bacteria*. Nevertheless, one cannot exclude that some of the detected sequences belong to microorganisms in dormancy. More extensive sequencing efforts will be required, that is, direct metatranscriptomics, to describe more directly the microbial communities along with functional signatures and to compile data on biogeochemical processes and fluxes.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

The ISME Journal

Supplementary Information

Ciobanu *et al*.

SI Text

Site description, sampling details and depth scale terminology. Hole U1352A was cored with the advanced piston corer system (APC; inside diameter: 6.2 cm) down to 42.2 m CSF-A. Hole U1352B was cored with the APC/extended core barrel (XCB; inside diameter: 6 cm) down to 830.9 m CSF-A. Hole U1352C was drilled and cored with the rotary core barrel (RCB; inside diameter: 5.87 cm) down to 1927.5 m CSF-A.

As a contamination control, micro-sized fluorescent microspheres were added during drilling (for every 10 m long sampled core) and the presence of microspheres was checked by epifluorescence microscopy in subsamples from the periphery to the inner center of each core sample. No contamination was detected in the center of cores analyzed in this study. Twenty-five whole-round sediment cores were subsampled onboard under sterile conditions. Subsamples were immediately frozen at –80°C for onshore molecular analyses. Subsamples were stored at 4°C under an anaerobic gas phase for later cultivation and sediment samples of 1 cm^3 were taken from the center of all whole-round samples and stored at 4°C in a 3% NaCl/3% formalin solution for onshore prokaryotic cell counting (for details see http://publications.iodp.org/preliminary_report/317/317PR.PDF) (Fulthorpe *et al.*, 2011). Rock samples for molecular analyses were also flamed in the laboratory before analysis.

Similarly, three holes were drilled at the neighboring site U1351 (44°53'04.22''S; 171°50'40.65"E) reaching a total depth of 967.3 m CSF-A and were subsampled for cell counting as reported elsewhere (Fulthorpe *et al.*, 2011).

Unless otherwise stated, all depth data in this paper refer to the core depth below seafloor computed by conventional method A (CSF-A) depth scale (see "IODP depth scales terminology" at www.iodp.org/program-policies/). This terminology is more accurate than that generally used in microbiological studies on sub-sea-floor samples as it takes into account sources of errors in depth scale determination such as incomplete recovery or core expansion/contraction. Nevertheless, since this method was also the one used in previous IODP studies, meters expressed in CSF-A are equivalent to mbsf (meters below seafloor) reported in the literature.

Cell count details. Samples from site U1351 were all counted directly onboard and some were also recounted in the laboratory. Samples from site U1352 were counted in the laboratory. As differences in cell counts were found between onboard data and later laboratory data with samples

from site U1351 a correction factor was calculated.

DNA extraction and PCR amplification details. DNA was extracted from at least 5 × 0.5 – 1 g uncontaminated frozen samples (–80°C) with the FastDNA™ Spin Kit for Soil (#6560-200, MP Biomedicals®), following the manufacturer's instructions. Ten μL of linear acrylamide (5 mg·mL^{-1}, Ambion/Applied Biosystems) were added to the protein lysis buffer in order to favor DNA precipitation in subsequent stages. At the final DNA extraction step, the DNA was eluted with 50 μL of DES solution. Additionally, DNA purifications were also made using a Microcon kit (Millipore) or on agarose gels. Whole genome DNA amplification attempts were also carried out using a commercial kit (GenomiPhi, GE Healthcare) with different DNA dilutions (1/10 and 1/100) and several amplification times (1.5 and 3 h). Concentration of extracted DNA was measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and PCR assays were performed on several dilutions: ½, 1/5, 1/10, 1/100 and 1/1000. In order to obtain direct positive PCR amplifications with our samples, a number of optimizations were performed. These included testing several DNA polymerases such as *Taq*CORE DNA polymerase (MP Biomedicals), Ampli*Taq* Gold® DNA polymerase (Applied Biosystems), TaKaRa™ Ex*Taq* DNA polymerase (Ozyme) and FastStart *Taq* DNA polymerase (Roche), according to the manufacturer's instructions. After comparison of PCR amplifications with different DNA polymerases, FastStart *Taq* DNA polymerase (Roche) was chosen.

All PCR mixtures (25 μL) contained 1× *Taq* DNA polymerase buffer with $MgCl_2$ (2 mM) (Roche), 1 mM of additional $MgCl_2$, 240 μM dNTP, 0.4 μM of each primer, 1 volume of 5× GC-rich buffer (Roche), 1 unit of FastStart *Taq* DNA polymerase (Roche) and 1 μL of DNA template. A "touchdown" PCR program was used to perform direct and nested PCRs for *Bacteria*, *Archaea* and *Eukarya* (Fig. S2). When the amplification methods listed above failed, DNA extracts were amplified with a nested PCR using several primer combinations (Table S2). For nested PCR amplifications targeting *Bacteria* and *Eukarya*, the touchdown gradient for the first PCR round was from 61 to 51°C and it was from 58 to 53°C for the second round. For archaeal nested PCR amplifications, the first "touchdown" PCR program was from 68°C to 58°C and the second touchdown PCR program was from 66°C to 61°C. For nested PCR amplifications, the reaction mixture was the same as that used for the first PCR amplifications (see above). In order to minimize stochastic PCR biases, two independent PCR products from the first round were pooled and used as template for the second PCR amplification (with 1 μL of mixed PCR products). This procedure was necessary to obtain visible PCR products on a 1% (w/v) agarose gel stained with ethidium bromide.

454-Pyrosequencing details. The sequencing of replicates was performed to overcome sequencing errors and to keep singletons retrieved in different pyrosequencing replicates, or in different samples (at different depths). For each sample, around 100 ng of PCR product was obtained and sent (on dry ice) to the Environmental Genomics facility of the *Observatoire des Sciences de l'Univers de Rennes* (University of Rennes I, France) where sequencing was performed.

Pyrosequencing was run on a Roche/454 Genome Sequencer FLX Titanium system (Roche). Design of fusion primer sequences containing Multiplex IDentifier (MID), emPCR amplification (GS FLX Titanium emPCR Kit Lib-A) and sequencing (GS FLX Titanium Sequencing Kit XLR70) were performed according to manufacturer's instructions. After image and signal processing with Roche software (gsRunProcessor v2.53) using default parameters, amplicons were subjected to the following steps of quality filtration using the trim.seqs command of the MOTHUR package (v. 1.23) (Schloss *et al.*, 2009) to remove sequences that (i) were shorter than 200 nucleotides or longer than 550 nucleotides, (ii) had one undetermined nucleotide or more, (iii) had homopolymers longer than six nucleotides, or (iv) had one or more errors in the primer sequence and/or in the MID sequences. Trim.seqs was also used to sort amplicons according to their MID sequences and to trim primer and MID sequences. For each sample, chimeric sequences were identified with the chimera.uchime command (MOTHUR v. 1.23) using reference=self. We did not use any denoising protocol. Technical replicates of PCR and sequencing were used to identify and eliminate spurious OTUs (Operational Taxonomic Units) that could inflate diversity estimates. OTUs were delineated at a 97% identity threshold using DNACLUST (Ghodsi *et al.*, 2011). In-house scripts were used to analyze sequence composition within each OTU and to select the OTUs gathering amplicons that had appeared independently in two or more replicates. In other words, OTUs composed of sequences originating from a single PCR amplification were discarded from the further analyses. In a few cases, negative controls of PCR turned positive. In consequence, a contaminant library was constructed from a pool of negative controls. This was the case for some bacterial and eukaryotic PCR negative controls. Contaminant sequences were included in the dataset to delineate OTUs and all OTUs containing at least one sequence from the negative controls were discarded. Richness and diversity indexes were computed from the list of OTUs, using MOTHUR (v. 1.23). To assign a taxonomic affiliation, amplicons were compared to the SILVA non-redundant set of small subunit sequences (SSURef_111_NR_tax_silva_trunc.fasta.tgz) (Quast *et al.*, 2013) and to the corresponding taxonomy using the classify.seqs command (MOTHUR v. 1.27).

Our pyrosequencing analysis was based on sediment samples uncontaminated by fluorescent microspheres. Despite the stringent conditions we used to avoid contaminants in our sequence sets, two OTUs were assigned to *Chrysophyceae* (golden algae) and four to taxa of *Magnoliophyta* (flowering plants) which are endemic to the Southern Hemisphere. These OTUs were present at four depth horizons. However only samples from the 346 m and 634 m CSF-A depth horizons showed a significant proportion of reads belonging to these groups. Although this finding caused us to question the results we obtained at these two depths, these phototrophic OTUs only contributed a tiny proportion of the eukaryotic reads at 583 m CSF-A (80 sequences out of 4,747) and 931m CSF-A (1 sequence out of 1,598). Such sequences were not found in the deepest samples. It is worth noting that none of these sequences were present in the negative control. To determine whether these sequences were fossil DNA, we processed alignments of plant sequences with close relative 18S rRNA gene sequences that displayed identity levels from 97 to 100%. Fossil

DNA is commonly characterized by abnormal substitutions or deamination of cytosine residues, but neither was observed in this case, suggesting that these sequences do not represent fossil DNA, even though this analysis was only processed on short sequences (454 reads). So, these sequences might correspond to contaminants. However, as kinetics of phosphodiester bonds have never been studied under pressure, and as plant sequences were observed only on these given horizons that were characterized by bioturbation, these sequences might also correspond to buried preserved remains (carbonates are good matrices for DNA preservation) or could have been transported to their positions during the last 500,000 years. Indeed, another recent study found rRNA signatures from plants and diatoms in marine sub-seafloor sediments ranging from 0.03 to 2.7 million years old (Orsi *et al*., 2013).

Real-time PCR measurement details. Bacterial and archaeal 16S rRNA gene copy numbers were tentatively converted to cell numbers by applying correction factors of 4.2 and 1.7, corresponding to the respective average copy numbers of 16S rRNA genes in these domains (source: rrndb http://rrndb.mmg.msu.edu, May 2013).

Cultures and approaches used for their analysis.

Microcolony cultivation in a sediment slurry membrane system. For the sediment slurry membrane cultivation system (Ferrari *et al.*, 2005, 2008), sediment slurry was prepared by adding 2-3 g of unsterilized marine sediment into the tissue culture insert (TCI) and then moistening it with 400 μL artificial seawater (ASW). TCIs were allowed to dry for five minutes and then placed upside down in a sterile six-well multidish (Nunc) that had been previously filled with 1 mL sterile Milli-Q water to prevent the TCIs from drying during incubations at high temperature (Fig. S8). Inocula were prepared by filtering 10 mL of a 1:100 sediment sample dilution on a 0.2 μm white polycarbonate membrane (PC) (Whatman), with a Büchner filtration device. The underside of an inoculated polycarbonate (PC) membrane was then placed on the TCI membrane (Fig. S8). The membrane system (TCI/PC membrane) allows nutrients and growth factors naturally present in the environment to be used by the microorganism community that grows on the PC membrane. The culture system (multidish containing TCI/PC membranes) was placed in an anaerobic jar containing two paper strips impregnated with a 10% (v/v) $Na_2S{\cdot}9H_2O$ solution, sealed, and incubated at 60-70°C for 2 weeks. Negative control preparations consisted of non-inoculated PC membranes that were placed on top of the TCI growth support system containing nonsterile sediment slurries. Each multidish contained a negative control (non-inoculated medium).

Media preparation and culture conditions. Each liter of artificial seawater (ASW), used for microcolony cultivation, contained: KBr (0.09 g), KCl (0.6 g), $CaCl_2$ (1.47 g), $MgCl_2.6H_2O$ (5.67 g), NaCl (30 g), $SrCl_2.6H_2O$ (0.02 g), $MgSO_4.7H_2O$, (5.62 g), NaF (0.002 g), and PIPES buffer (3 g). The pH was adjusted to 7.5. After autoclaving, the medium was cooled and the following solutions were added from sterile stock solutions: NH_4Cl (4.67 mM), KH_2PO_4 (1.5 mM), a solution of seven vitamins (1×) and a trace element solution (1X). Different anaerobic metabolisms were targeted in culture: fermentation, sulfate-reduction and methanogenesis/acetogenesis. For the enrichment culture step, all media

contained 10 μM cAMP (Cyclic adenosine monophosphate), a cellular signalization molecule, and were inoculated as described above. Fermentation assays were performed on samples 137R and 148R; fermentation media were supplemented with 0.5 $g \cdot L^{-1}$ yeast extract, 0.5 $g \cdot L^{-1}$ peptone and 0.5 $g \cdot L^{-1}$ casamino acids. For methanogens/acetogens, four types of substrate were used: (1) H_2/CO_2 80/20 (2 bars), (2) 10 mM acetate, (3) ethanol/methanol, 0.5% (v/v) (and incubation under an atmosphere of N_2/CO_2 80/20 (2 bars)), and (4) a mixture of methylamine and formate at a concentration of 10 mM each. Enrichment cultures of methanogens/ acetogens were performed with samples 137R and 148R. All media contained resazurin (0.5 $g \cdot L^{-1}$) as an anaerobiosis indicator, and were incubated for two weeks at 70°C in the dark. Fungal cultures were prepared in Potato Dextrose Broth (Sigma) saturated with dissolved oxygen, and either supplemented with 3% (w/v) sea salts (PDB3) or not (PDB0). Replicates were processed for each positive sample (3H5, 5H3, 88X1). Sample 3H5 was incubated in plastic tubes sealed with sterile Parafilm at 4 MPa and 25°C, 5H3 and 88X1 at 11 MPa and 25°C. Cultivation under high-hydrostatic pressure was carried out in 10 mL syringes loaded with medium and then placed into a high-pressure/high temperature incubation system, custom-built by Top-Industrie (Vaux le Pénil, France).

Microcolony visualizations, microscopic observations, fluorescent in situ hybridizations. Growth on PC membranes was checked by fluorescence microscopy after staining of total cells on PC membranes. A quarter of each PC membrane was stained with SYBR®Green I (1:1000, Invitrogen, Molecular Probes), as follows: 20 μL of a 10× SYBR®Green I solution was deposited on a PC membrane section, incubated in the dark for about 15 minutes, air dried and directly mounted on a slide with 40 μL anti-fading solution (50% glycerol, 50% PBS [120 mM NaCl, 10 mM NaH_2PO_4 pH 7.5], 0.1% *p*-phenylenediamine). Microcolonies were observed under immersion with an Olympus BX60 epifluorescence microscope (objective 100×, pH3, WIB filter). Images were processed with QCAPTURE PRO 6.0 software. Prokaryotic cells in liquid subcultures were regularly observed by light and epifluorescence microscopy. Growth was monitored by epifluorescence microscopy after culture filtration (0.02 μm pores) and staining with SYBR®Green I (Olympus BX60; magnification 100×, pH3, WIB filter). For observations by fluorescence *in situ* hybridization, fungal cultures were fixed with 4% (w/v) paraformaldehyde for 3 hours at 4°C. After fixation, cultures were washed three times in phosphate buffer saline and stored in phosphate buffer saline/96% ethanol (v/v) solution. Forty μL of this solution was put on a slide. After drying for 20 min at 46°C, each slide was covered with a 0.1% (w/v) agarose layer. Cells were then dehydrated in three successive baths of ethanol of increasing concentration (50%, 80% and 96%). Hybridization was performed with hybridization buffer (NaCl 0.9M, Tris-HCL 10.18M, SDS 0.01%, formamide 20%) and 3 ng/μL of oligonucleotide probe Euk516 labeled with the fluorochrome Cy3. In addition, 1 μL DAPI was introduced in order to visualize the cell nucleus. After 3 hours of incubation at 46°C, each slide was rinsed independently in a unique buffer rinsing (20 mM Tris-HCl, 5 mM EDTA and 215 mM NaCl) for 10 min at 48°C. The slides were then observed by epifluorescence microscopy (Olympus BX51; magnification X60; Olympus UPlanFL N).

Liquid enrichment subcultures. When microcolonies were detected on a (PC) membrane, a quarter section of the same PC membrane was transferred to a new sterile vial containing 5 mL of enrichment medium (ASW supplemented with given substrates) and then incubated at 60-70°C (Fig. S6). Growth was regularly checked by photonic and epifluorescence microscopy (staining with 5 μL 10× SYBR®Green I solution for 100 μl culture). Following at least 30 days of incubation, positive cultures were transferred (1:50 inoculation) under the same conditions to a new sterile vial containing 20 mL of the same medium. Subsequently, given the slow growth and the extremely low biomasses observed in the first subcultures in liquid media, sterile glass beads and a polycarbonate filter were added to the vials in order to allow the microorganisms to adopt an "attached lifestyle", as in the first enrichment system (Fig. S8). From then, cultures were conducted in the manner of a culture media renewal. To perform culture media renewal in subsequent liquid subcultures, almost all the culture volume was removed aseptically under anaerobic conditions and stored at 4°C in a sterile anaerobic vial, under an atmosphere of 100% N_2. Only 0.5 mL of culture was kept in the vial with the original growth media (plus the glass beads and the polycarbonate filter) and then, 20 mL of fresh medium was added to the original vial, using a syringe. The vials were re-labeled accordingly. In order to stimulate growth and to supply growth factors in liquid subcultures, 0.1 $g \cdot L^{-1}$ of yeast extract was also added. This was done, as it appeared that we did not enrich true methanogens and true sulfate-reducers in the media targeting these physiological groups, but rather strains degrading yeast extract or PIPES buffer.

With mean cell densities around 4.5×10^5 $cells \cdot mL^{-1}$, growth was difficult to monitor. Only the fact that the density in the cultures was maintained after 6-9 subcultures inoculated at 1/40 or 1/50 formally demonstrated that growth occurred in these cultures. The overall dilution factor of the original sample [F(y)] in different subcultures can be calculated as follows: $F(y) = [(20/0.4)^y] \times [(20/0.5)^5]$ where **y** represents the number of subcultures made with classic transfer (= inoculation of a fresh medium; depending on the sample, the classic transfer method was used from 1 to 4 times) and 5 is the number of subcultures by simple renewal of the medium. Thus, the samples were diluted at least $F(1) = 2 \times 10^9$ times and a maximum of $F(4) = 2 \times 10^{12}$ times. Cell counts made on crude sediments from the Canterbury Basin have indicated abundances around 3×10^4 cells/mL. Consequently, considering the wide overall sample dilution factor of the original sample after 6 to 9 subcultures and the observation that that were still 10^3 to 10^5 cells/mL in the last subcultures, there are strong indications that growth occurred within cultures. Altogether, the small cell-sizes, the low abundances and the very slow growth rates made these community cultures very difficult to study. After two and a half years of cultivation and 6 to 9 subcultures, these low-density and slow-growing community cultures of fermentative bacteria could not be subcultured further, probably because nutritional or physical-chemical conditions were not optimal.

ATP quantification and viability assays. Cell viability and structural integrity in bacterial liquid subcultures was monitored using the dual staining LIVE/DEAD® Bacterial Viability Kit (BacLight™) (Molecular Probes), following the manufacturer's instructions. ATP content

within cultures was determined using the Bac Titer-Glo Microbial Cell Viability Assay (Promega) with a 100 nM ATP standard.

DNA/RNA extractions. DNA extractions from liquid subcultures were performed by the phenol-chloroform-isoamyl alcohol method. Five mL of culture were centrifuged (in 3 times) in an Eppendorf at 4°C for 45 min at 13000 x *g*, and the cell pellet was extracted and eluted in 40 μL DEPC water, then concentrated with a kit Microcon YM-total 100 (Millipore) according to manufacturer's instructions. The concentrated DNA was eluted in 50 μL ultrapure water. As a negative control, a DNA extraction with sterile Milli-Q water was also performed. For RNA extractions, the cell pellet (see above) was suspended in 0.6 mL of STE buffer [STE buffer (per 100 ml): 1 mL Tris / HCl 1 M, pH 8.3, 200 μL EDTA 0.5 M, pH 8.0, 2 mL NaCl 5 M, 96.8 mL RNase-free water] and 1.08 mL of hot acid phenol (pH 4.5 to 5.0; Roth) preheated to 60°C in a water bath. Subsequently, 240 μL SDS 10% (v/v) were added and the mix was incubated in a water bath for 8 min at 60°C. Tubes were then placed on ice and centrifuged 4°C for 25 min at 5000 x *g*. After centrifugation, the upper aqueous phase was recovered and transferred to a new Eppendorf tube. Eighty μL of sodium acetate (3M) and a volume of hot phenol were added and the tubes were centrifuged again (see above). At this step, the upper aqueous phase was recovered in a new tube and a volume of PCI (125:24:1, pH 4.5 to 5.0; Roth) was added. The solutions were mixed by gentle inversion and then centrifuged. The upper aqueous phase was then recovered and one volume of chloroform was added. The tubes were then centrifuged, and the aqueous phase was recovered without disturbing the interface. RNA was precipitated by adding 2.5 volumes of 96% (v/v) ethanol to the aqueous phase (30 min at −80°C). RNA was pelleted by centrifugation (1 h at 4°C, 5000 x *g*). As a final step, the pellet was washed with 70% (v/v) ethanol and suspended in 50 μL RNase-free water. A DNase treatment was performed by adding 6 μL 10× DNase buffer (Fermentas), 0.6 μL DTT (0.1 mol.L^{-1}; Roche), 0.6 μL RNase inhibitor (40 units/μL; Roche), 3 μL DNase (1 unit/μL), and incubating the mixture at 37°C for 1.5 h. The reaction was stopped by adding 6 μL EGTA, 20 mM, pH 8. A negative control of RNA extraction was performed with sterile Milli-Q water.

PCR and RT-PCR amplifications. 16S rRNA gene sequences were amplified from the DNA/RNA extracts, and from negative controls, using the universal bacterial primers Bac8F/1492R and the *Taq* core DNA polymerase (MP Biomedicals), following the manufacturer's amplification protocol. For the amplifications from RNA extracts, a RT-PCR was performed with 11 μL RNA with the kit RevertAid™ H Minus First Strand cDNA Synthesis (Fermentas), using the primer 1492R 5'−ACGGHTACCTTGTTACGACTT−3', following the manufacturer's instructions. Then, a PCR was performed with the *Taq* core DNA polymerase (MP Biomedicals). RT-PCR and direct PCR negative controls were also performed on RNA extracts to confirm the absence of contaminant DNA.

Nucleic acid purification. Prior to cloning, PCR products were purified on gel to remove dNTPs or potential non-specific bands. PCR products were migrated on a 1% (w/v) agarose gel and bands of interest were extracted and purified with the kit QIAEX II Agarose Gel Extraction (Qiagen®), according to manufacturer's instructions. Purified DNA was resuspended in 35 μL sterile Milli-Q water

and stored at 4°C. The DNA quantification obtained after purification was evaluated by deposition and migration of 5 μL of purified product on a 1% (w/v) agarose gel.

Cloning, sequencing and sequence analysis. Prior to cloning, PCR amplicons stored at 4°C were given a poly-A tail by incubating the PCR mix (see above) at 72°C for 10 min, then cooling it to 4°C for another 10 min. Four μL of PCR product were then cloned with the TOPO®TA Cloning kit (Invitrogen™), following the manufacturer's recommendations. Ligation time was extended to 30 min and transformation (within TOP 10 10F' competent cells) was done within 2 h under gentle shaking (200 rpm). Cells were then plated onto LB agar plates (NaCl, 10g·L^{-1}, tryptone 10 g·L^{-1}, yeast extract 5 g·L^{-1}, agar 20 g·L^{-1}, pH 7.5, ampicillin 50 mg·L^{-1}). One hundred colonies were picked randomly from the transformation plates and grown in deep-wells containing 1.5 mL of LB medium supplemented with ampicillin at 50 mg·L^{-1}. Deep-well plates were incubated at least 24 hours at 37°C. For each clone culture, a volume of 200 μL was stored in 25% (v/v) glycerol at -20°C. Plasmids were extracted by an alkaline lysis method in deep-wells. Plasmid DNAs were resuspended in 50 μL 1× TE buffer. PCR products were amplified from cloned rRNA gene fragments using primers M13F and M13R, and sequenced. PCR amplicon sequencing was carried out by the Cogenics-Beckman Coulter Genomics Company. Sequences were compared to those present in the databases using the BLASTn algorithm (and then aligned to their nearest neighbor using the programs CLUSTAL_X v2 and PHYLIP v3.69).

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Supplementary Information

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SI Tables

Table S1 List of studied samples and depths

Sample name	Core depth below the seafloor (m)
1H3	4
2H1*	6
3H1*	15
3H5	21
4H1	25
4H5*	31
5H3	37
21H3	184
42X*	346
48X	403
61X*	524
68X*	583
73X*	634
81X	709
88X1	765
34R*	931
41R	1003
87R*	1367
105R*	1524
110R*	1577
122R*	1690
125R*	1711
128R*	1740
137R*	1827
147R*	1911
148R*	1922

*Samples analyzed by using 454-pyrosequencing of 6S/18S rRNA gene-tagged amplicons and real-time PCR.

Table S2 Primers used in this study

Primer	Target	Sequence* (5' – 3')	Tm
Bac 341F	*Bacteria* 16S rRNA	CCTACGGGAGGCAGCAG	58°C
Uni 907R	*Bacteria* 16S rRNA	CCGTCAATTCMTTTGAGTTT	50°C
Bac AF†	*Bacteria* 16S rRNA	GTGCCAGCMGCCGCGGTAATAC	64°C
Bac BR†	*Bacteria* 16S rRNA	CCGTCAATTCCTTTGAGTTT	51°C
Bac 8F	*Bacteria* 16S rRNA	AGAGTTTGATCATGGCTCAG	58°C
1492R	*Bacteria* 16S rRNA	ACGGHTACCTTGTTACGACTT	50°C
Arc 27Fa†	*Archaea* 16S rRNA	TCYGGTTGATCCTGSCGG	58°C
Arc 806R	*Archaea* 16S rRNA	GGACTACVSGGGTATCTAAT	50°C
Arc 519R†	*Archaea* 16S rRNA	GGTDTTACCGCGGCKGCTG	61°C
Euk 82F	*Eukarya* 18S rRNA	GAADCTGYGAAYGGCTC	52°C
Euk 516R†	*Eukarya* 18S rRNA	ACC AGACTTGCCCTCC	54°C
Euk 42F†	*Eukarya* 18S rRNA	CTCAARGAYTAAGCCATGCA	53°C

*Degenerated primers: R=G/A, Y=T/C, M=A/C, K=G/T, S=G/C, W=A/T, H=A/C/T, B=G/T/C, V=G/C/A, D=G/A/T, N=G/A/T/C.
†Primers used for pyrosequencing.

Table S3 Fusion primers used in this study

Name	Primer sequence (5'-3')[§]
***Bacteria*, Bac AF/Bac BR**	
P-16RM13	[†]CGTATCGCCTCCCTCGCGCCATCAGCTCGACACCGTCAATTCCTTTGAGTTT
P-16FM13	[‡]CTATGCGCCTTGCCAGCCCGCTCAGTGATACGTGTGCCAGCMGCCGCGGTAATAC
P-16RM14	CGTATCGCCTCCCTCGCGCCATCAGTCACGTACTACCGTCAATTCCTTTGAGTTT
P-16FM14	CTATGCGCCTTGCCAGCCCGCTCAGACTACTATGTGCCAGCMGCCGCGGTAATAC
P-16RM15	CGTATCGCCTCCCTCGCGCCATCAGAGACTATACTCCGTCAATTCCTTTGAGTTT
P-16FM15	CTATGCGCCTTGCCAGCCCGCTCAGACGCTATGTGCCAGCMGCCGCGGTAATAC
***Eukarya*, Euk 82F/Euk 516R**	
EukRX1	CGTATCGCCTCCCTCGCGCCATCAGCACGCTACGTACCAGACTTGCCCTCC
EukFX1	CTATGCGCCTTGCCAGCCCGCTCAGTACACGTGATGAADCTGYGAAYGGCTC
EukRX2	CGTATCGCCTCCCTCGCGCCATCAGTACAGATCGTACCAGACTTGCCCTCC
EukFX2	CTATGCGCCTTGCCAGCCCGCTCAGTAGTGTAGATGAADCTGYGAAYGGCTC
EukRX3	CGTATCGCCTCCCTCGCGCCATCAGTCGATCACGTACCAGACTTGCCCTCC
EukFX3	CTATGCGCCTTGCCAGCCCGCTCAGTCTATACTATGAADCTGYGAAYGGCTC
EukRX4	CGTATCGCCTCCCTCGCGCCATCAGAGCTCACGTAACCAGACTTGCCCTCC
EukFX4	CTATGCGCCTTGCCAGCCCGCTCAGAGTATACATAGAADCTGYGAAYGGCTC
EukRX5	CGTATCGCCTCCCTCGCGCCATCAGCGTACTCAGAACCAGACTTGCCCTCC
EukFX5	CTATGCGCCTTGCCAGCCCGCTCAGCTCGCGTGTCGAADCTGYGAAYGGCTC
EukRX6	CGTATCGCCTCCCTCGCGCCATCAGCTACGCTCTAACCAGACTTGCCCTCC
EukFX6	CTATGCGCCTTGCCAGCCCGCTCAGCGAGAGATACGAADCTGYGAAYGGCTC
EukRX7	CGTATCGCCTCCCTCGCGCCATCAGTAGTCGCATAACCAGACTTGCCCTCC
EukFX7	CTATGCGCCTTGCCAGCCCGCTCAGTCGTGACATGGAADCTGYGAAYGGCTC
EukRX8	CGTATCGCCTCCCTCGCGCCATCAGAGTCAGTAACCAGACTTGCCCTCC
EukFX8	CTATGCGCCTTGCCAGCCCGCTCAGCATACTCTACGAADCTGYGAAYGGCTC
EukRX9	CGTATCGCCTCCCTCGCGCCATCAGTGTCGTCGCAACCAGACTTGCCCTCC
EukFX9	CTATGCGCCTTGCCAGCCCGCTCAGTGCTATAGACGAADCTGYGAAYGGCTC
EukRX10	CGTATCGCCTCCCTCGCGCCATCAGACAGTCGTGCACCAGACTTGCCCTCC
EukFX10	CTATGCGCCTTGCCAGCCCGCTCAGATGTGTCTAGGAADCTGYGAAYGGCTC
EukRX11	CGTATCGCCTCCCTCGCGCCATCAGCGACACTATCACCAGACTTGCCCTCC
EukFX11	CTATGCGCCTTGCCAGCCCGCTCAGCACTCGCACGGAADCTGYGAAYGGCTC
EukRX12	CGTATCGCCTCCCTCGCGCCATCAGTAGACTGCACACCAGACTTGCCCTCC
EukFX12	CTATGCGCCTTGCCAGCCCGCTCAGCTCGATATAGGAADCTGYGAAYGGCTC
EukRX13	CGTATCGCCTCCCTCGCGCCATCAGCTGCGTCACGACCAGACTTGCCCTCC
EukFX13	CTATGCGCCTTGCCAGCCCGCTCAGCGTGATGACGAADCTGYGAAYGGCTC
EukRX14	CGTATCGCCTCCCTCGCGCCATCAGCTCTACGCTCACCAGACTTGCCCTCC
EukFX14	CTATGCGCCTTGCCAGCCCGCTCAGTATAGACATCGAADCTGYGAAYGGCTC
EukRX15	CGTATCGCCTCCCTCGCGCCATCAGAGTGCTACGAACCAGACTTGCCCTCC
EukFX15	CTATGCGCCTTGCCAGCCCGCTCAGCGTGTCTCTAGAADCTGYGAAYGGCTC
EukRX16	CGTATCGCCTCCCTCGCGCCATCAGAGACTCAGCGACCAGACTTGCCCTCC
EukFX16	CTATGCGCCTTGCCAGCCCGCTCAGTGAGCTAGAGGAADCTGYGAAYGGCTC
***Archaea*, Arc 27Fa/Arc 519R**	
ArcFY1	CTATGCGCCTTGCCAGCCCGCTCAGTAGACTAGTCYGGTTGATCCTGSCGG
ArcRY1	CGTATCGCCTCCCTCGCGCCATCAGGCTGTCTGGTDTTACCGCGGCKGCTG
ArcFY2	CTATGCGCCTTGCCAGCCCGCTCAGAGTAGTGATCTCYGGTTGATCCTGSCGG
ArcRY2	CGTATCGCCTCCCTCGCGCCATCAGACAGTATATAGGTDTTACCGCGGCKGCTG
ArcFY3	CTATGCGCCTTGCCAGCCCGCTCAGACTCGCGCACTCYGGTTGATCCTGSCGG
ArcRY3	CGTATCGCCTCCCTCGCGCCATCAGACACATACGGTDTTACCGCGGCKGCTG
ArcFY4	CTATGCGCCTTGCCAGCCCGCTCAGACGTGCAGTCYGGTTGATCCTGSCGG
ArcRY4	CGTATCGCCTCCCTCGCGCCATCAGCTCTATCGGTDTTACCGCGGCKGCTG
ArcFY5	CTATGCGCCTTGCCAGCCCGCTCAGTCACTCATACTCYGGTTGATCCTGSCGG
ArcRY5	CGTATCGCCTCCCTCGCGCCATCAGCTCGCTAGGTDTTACCGCGGCKGCTG

[†]Adapter A ; [‡]Adapter B ; [§]adapter/key/MID/primer
MID : Multiplex identifier

Table S4 Sequencing information

	Bacteria	*Archaea*	*Eukarya*
Primer sets used for 454-pyrosequencing	Bac AF/Bac BR	Arc 27Fa/Arc 519R	Euk42F/Euk516R
Target regions of the 16S/18S rRNA gene	V4-V5	V1-V3	V1-V3
Average length of the target region (bp)	411	509	449
Total number of tag sequences*	17803	9979	24908
Total OTUs at 3% difference	198	16	40

*Trimmed reads that passed quality controls

Table S5 Vertical distribution of OTUs at 3% divergence that occurred more than 100 times

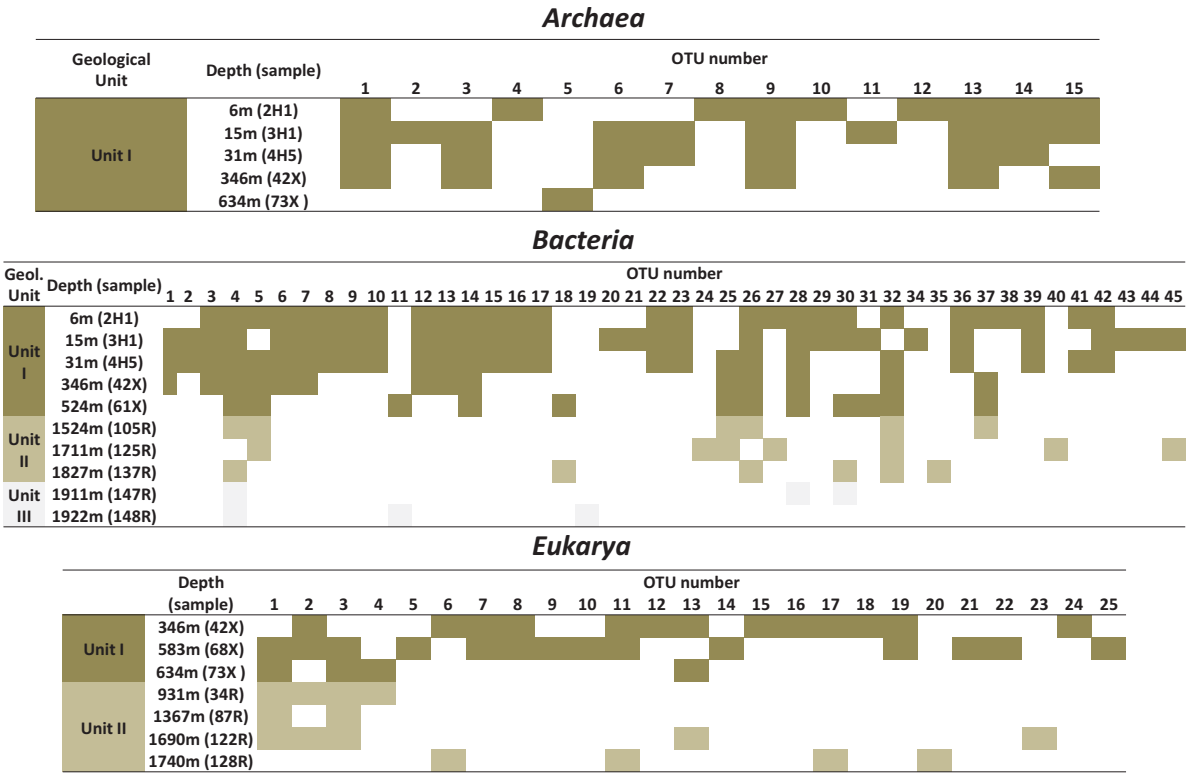

Table S6 Molecular identification of the fungal strain from sample 88X1 based on the ITS1 region of rRNA

Identity / Alignment	Fungal strain 88X1	*Cadophora malorum* (JQ796752)	*Cadophora malorum* (GU004209)	*Cadophora fastigiata* (JN942894)
Fungal strain 88X1	1	237/238	237/238	237/238
***Cadophora malorum* (JQ796752)**	0.99	1	593/593	567/567
***Cadophora malorum* (GU004209)**	0.99	1	1	567/567
***Cadophora fastigiata* (JN942894)**	0.99	1	1	1

SI Figures

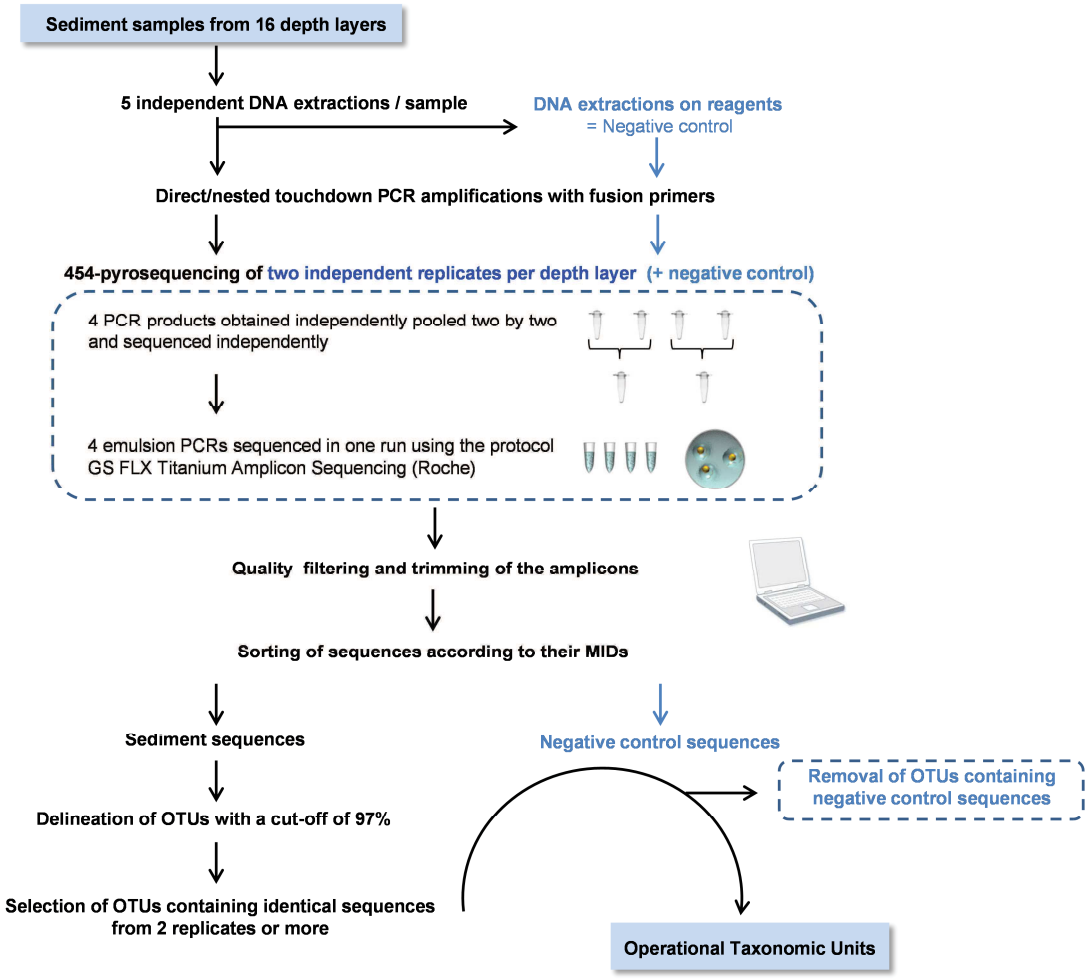

Figure S1 Flow-diagram of the 454-pyrosequencing strategy.

(A) **"Touchdown" PCR**

95°C	7 min	
95°C	1 min	
*61°C	1 min	X 20[§]
72°C	1.5 min	
95°C	1 min	
*51°C	1 min	X 16[§§]
95°C	1.5 min	
72°C	7 min	

Bacterial primer sets
Direct PCR: BacAF/BacBR, [§:§§]20:16 cycles
Nested PCR: Bac341F/Uni907R, [§:§§]20:7 cycles; BacAF/BacBR, [§:§§]10:9 cycles

Archaeal primer sets
Direct PCR: ARC27Fa/ARC519R, [§:§§]20:16 cycles
Nested PCR: ARC27Fa/ARC806R, [§:§§]20:7 cycles; ARC27Fa/ARC519R, [§:§§]10:9 cycles

Eukaryotic primer sets
Nested PCR: Euk42F/Euk516R, [§:§§]20:7 cycles; Euk82F/Euk516R, [§:§§]10:9 cycles

(B) **Fusion primer configuration**

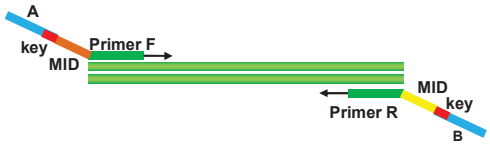

Figure S2 A. "Touchdown" PCR programs used for direct and nested PCR amplifications, and primer pairs used for each domain. **B**. Diagram of general fusion primer configuration. Direct PCR amplifications were done with fusion primers specifically designed for each domain. Nested PCR amplifications were performed with "normal" primers for the first round and with fusion primers for the second round.

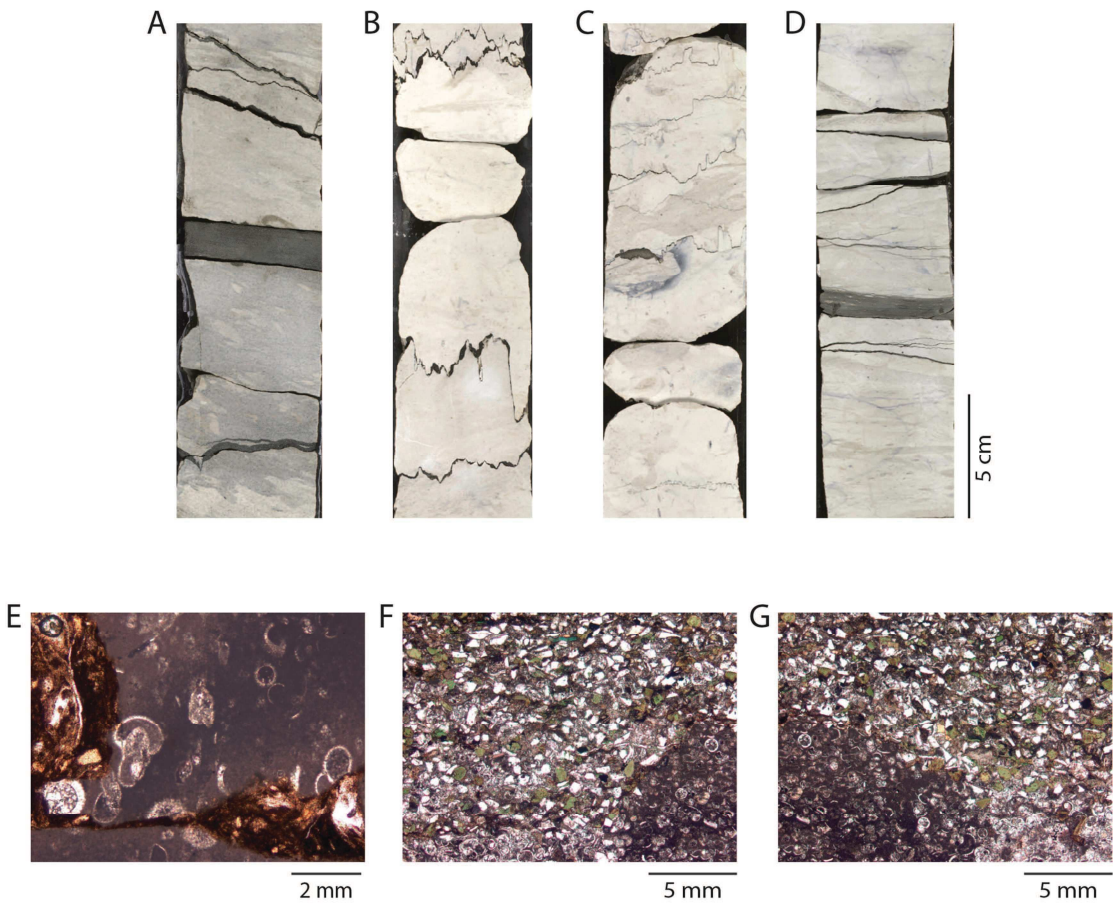

Figure S3 Core photographs of contact between Units II and III, and thin section photomicrographs illustrating changes in lithology and diagenesis in Units II and III. **A**. Glauconitic limestone above the unconformity, Subunit IIC (interval 317-U1352C-140R-1, 6–26 cm). **B**. Stylolitic white limestone below the unconformity, Unit III (interval 317-U1352C-141R-2, 14–34 cm). **C**. Stylolitic white limestone with purple banding and pyrite concentrated in a stylolite, Unit III (interval 317-U1352C-144R-2, 41–61 cm). **D**. Muddy limestone with layers of marlstone and purple banding, Unit III (interval 317-U1352C-148R-1, 62–68 cm). **E**. Section of a high-amplitude stylolite passing through pelagic limestone with sparse foraminifers in Unit III. The stylolite is marked by brownish clay, along which the foraminifers are truncated (Sample 317-U1352B-141R-2, 15 cm). **F**, **G**. Abrupt high-angle contact between muddy limestone to limestone (transitional) below (within host rock) and glauconitic sandy marlstone above (potential injection material) in Unit II. Limestone color is a function of the foraminifer/nannofossil ratio (higher in light limestone; lower in dark limestone) (Sample 317-U1352C-137R-2, 58 cm). (Source: IODP report 317)(Fulthorpe *et al*., 2011).

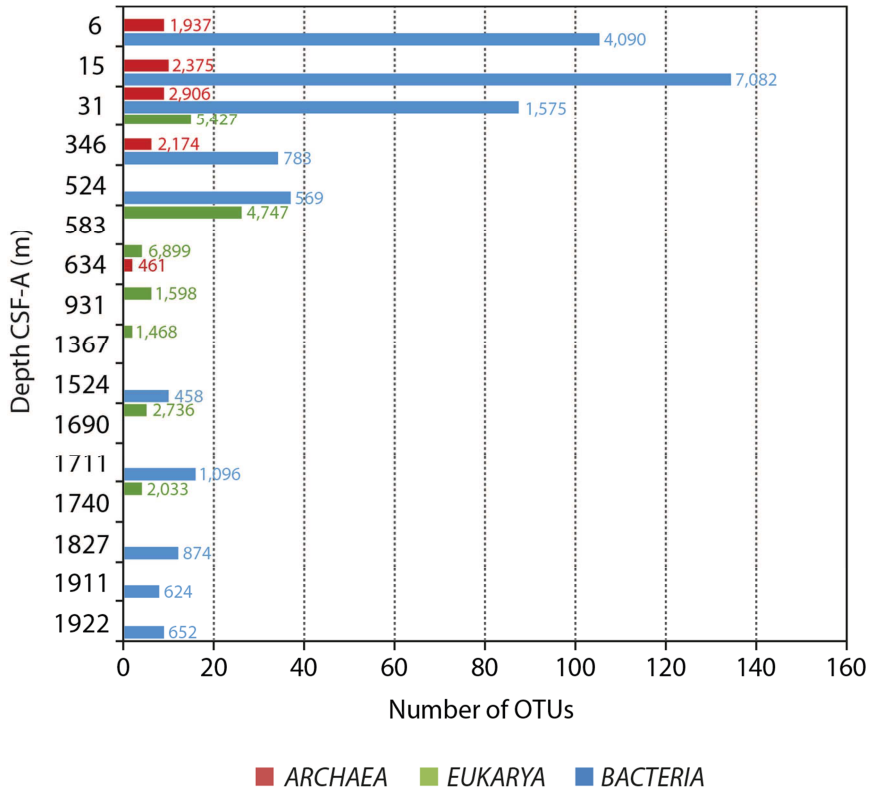

Figure S4 Number of OTUs found for *Archaea* (red bars), *Eukarya* (green bars) and *Bacteria* (blue bars) for each depth sample. OTUs were defined using a strict 3% dissimilarity threshold between 16S or 18S rDNA gene sequences. Numbers indicated to the right of each bar correspond to tag sequences constituting OTUs that were obtained by 454-pyrosequencing for each domain and depth sample.

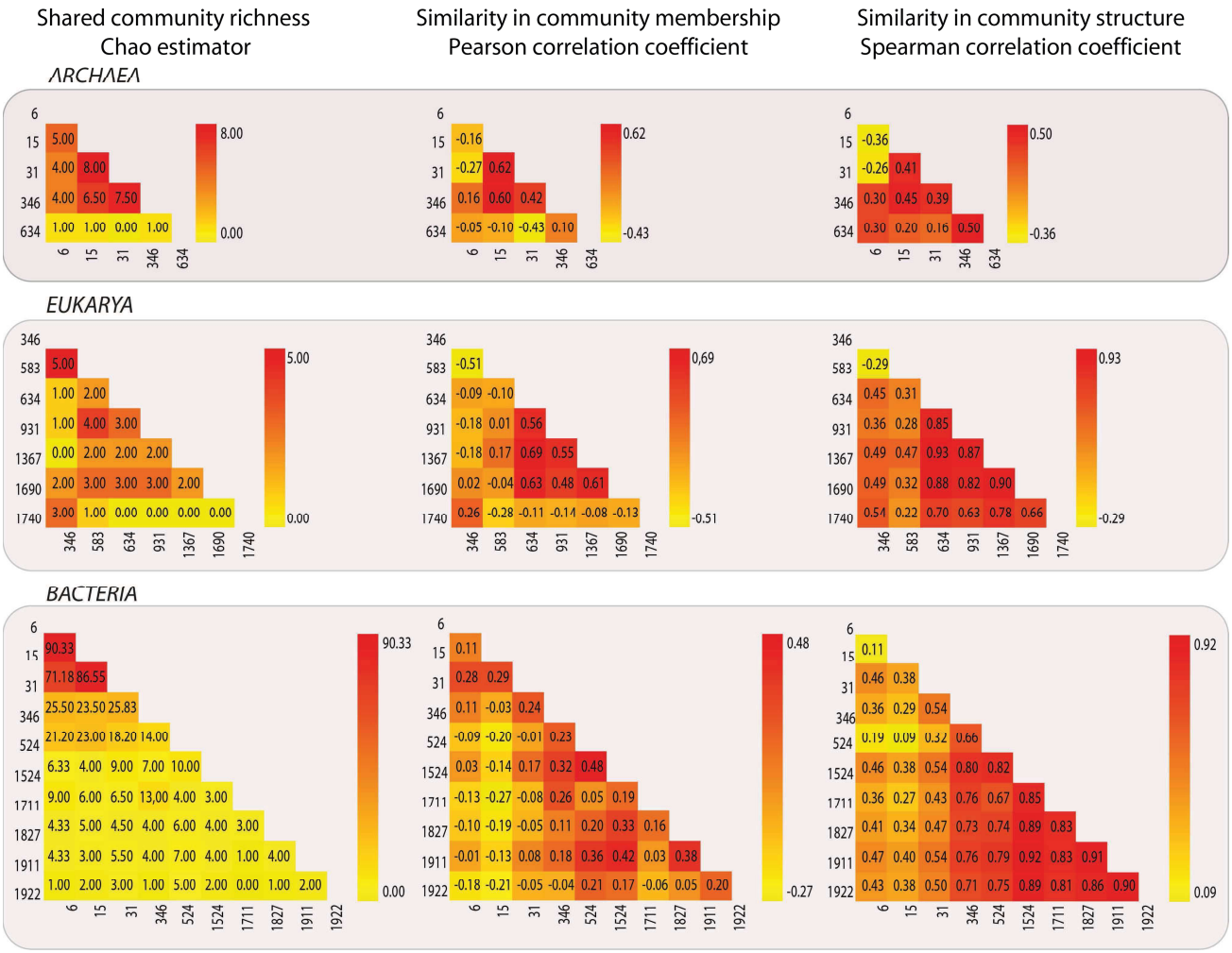

Figure S5 Heat-map matrixes of β-diversity indices calculated by MOTHUR at 3% difference between OTUs. The index score indicates the similarity between two bacterial, archaeal or eukaryotic communities: Chao estimator for shared community richness; Pearson correlation coefficient for assessment of similarity in community membership; Spearman correlation coefficient for assessment of similarity in community structure.

Pearson correlations (r) between environmental parameters and their significance based on *p-values*

NS	not significant
*	0.01<P<0.05 (light grey)
**	<0.01 (dark grey)
***	<0.001 (dark)

PARAMETERS	Depth	Calcium carbonate	Inorganic carbon	Organic carbon	Methane	Ethane	Alkalinity	Ammonium	Calcium	Magnesium	pH	Salinity	Sulfate	Porosity
Depth	**1**	***	***	NS	NS	**	**	NS	**	NS	NS	**	NS	***
Calcium carbonate	**0,943**	**1**	***	NS	NS	**	NS	NS	NS	**	NS	NS	NS	***
Inorganic carbon	**0,943**	**1,000**	1	NS	NS	**	NS	NS	NS	**	NS	NS	NS	***
Organic carbon	0,019	-0,061	-0,061	1	NS	NS	NS	*	NS	**	NS	NS	NS	NS
Methane	-0,382	-0,310	-0,310	-0,217	1	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ethane	**0,656**	**0,710**	**0,710**	-0,112	-0,143	1	NS	NS	NS	NS	NS	NS	NS	**
Alkalinity	**-0,860**	-0,439	-0,439	-0,352	0,554	-0,266	1	NS	***	NS	NS	NS	NS	NS
Ammonium	0,461	0,627	0,627	**0,674**	-0,217	0,583	-0,309	1	NS	**	NS	**	NS	NS
Calcium	**0,851**	0,288	0,288	0,201	-0,355	0,061	**-0,879**	-0,003	1	NS	NS	NS	NS	*
Magnesium	-0,541	**-0,846**	**-0,846**	**-0,871**	0,192	-0,353	0,237	**-0,893**	-0,030	1	NS	*	*	NS
pH	0,005	0,015	0,015	0,254	-0,116	0,157	0,144	-0,376	-0,025	0,105	1	NS	NS	NS
Salinity	**-0,736**	-0,541	-0,541	-0,479	0,194	-0,406	0,565	**-0,821**	-0,488	**0,701**	0,502	1	*	NS
Sulfate	-0,453	-0,522	-0,522	-0,525	-0,111	0,481	0,016	-0,600	-0,129	**0,720**	0,242	**0,756**	**1**	NS
Porosity	**-0,968**	**-0,942**	**-0,942**	0,073	0,318	**-0,645**	0,500	0,112	**-0,686**	0,201	-0,401	0,238	0,245	**1**

Network of correlations between the 14 environmental parameters characterizing IODP Leg317 sediment core. Inner box parameters are proxys for outer box ones.

Positive correlation

Negative correlation

***

**

*

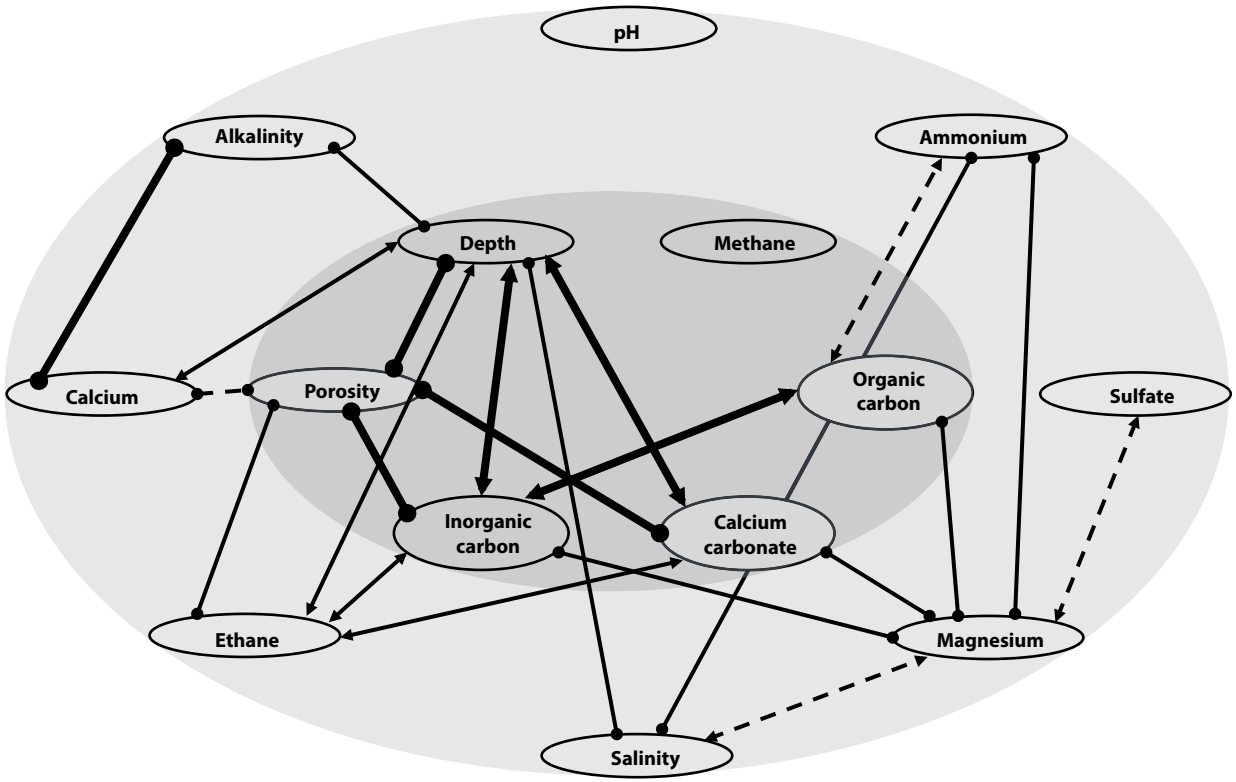

Figure S6 Relationships between environmental parameters based on Pearson correlations (r). A network of correlations was built based on both positive and negative correlations. Depth, Methane, Organic carbon, Calcium carbonate, Inorganic carbon and Porosity were chosen as proxies for Ammonium, Sulfate, Magnesium, Salinity, Ethane, Calcium and Alkalinity.

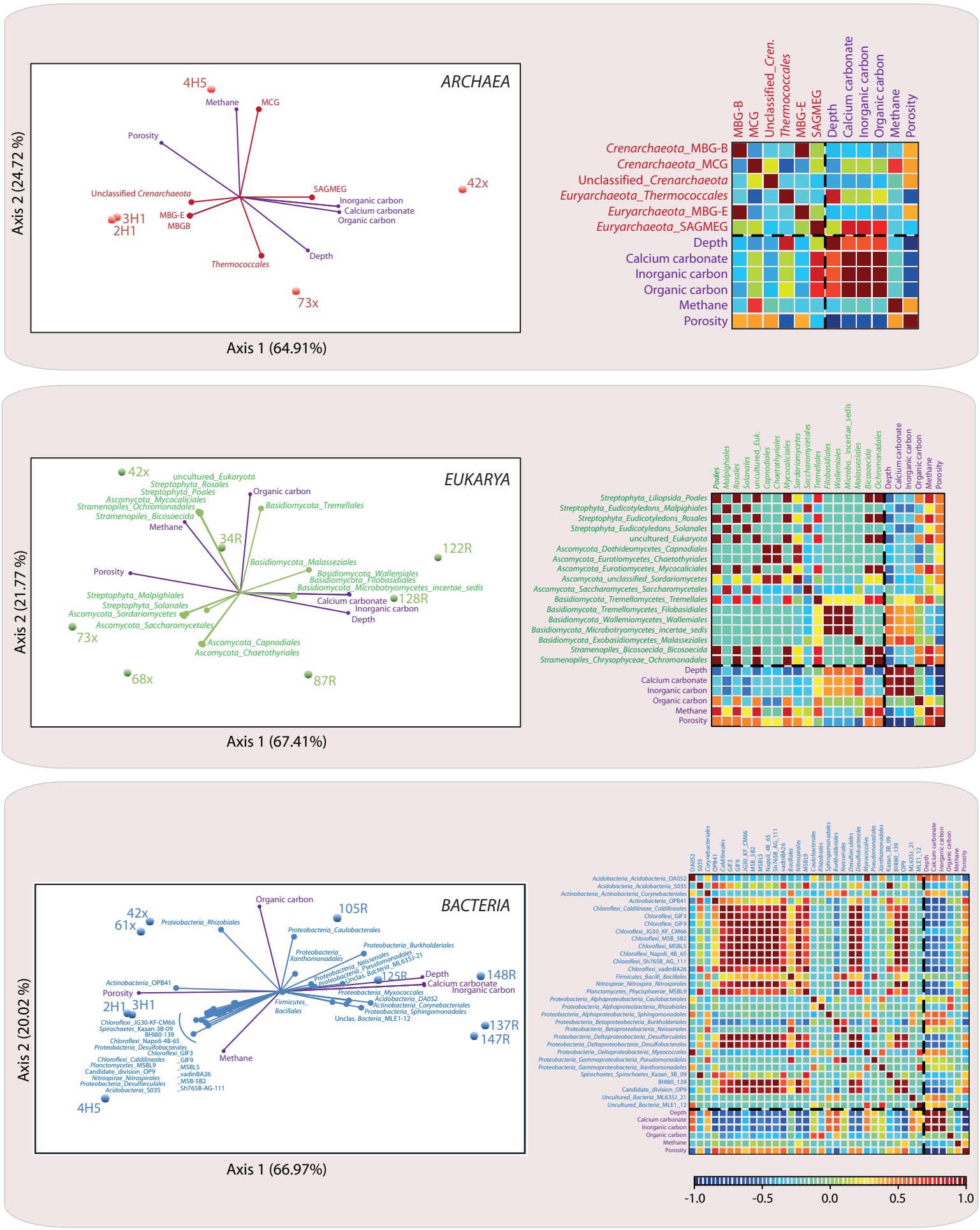

Figure S7 Relationships between order abundances of archaeal, eukaryotic and bacterial 16S/18S rRNA genetag sequences and selected environmental parameters. (Left part) Principal Component Analyses (PCA) with sediment samples (black dots), microbial taxa (blue vectors) and environmental parameters (red vectors). (Right part) Correlation matrices of regularized canonical correlation analysis (RCCA) with a color scale, indicating positive correlation values in red tones and negative correlation values in blue.

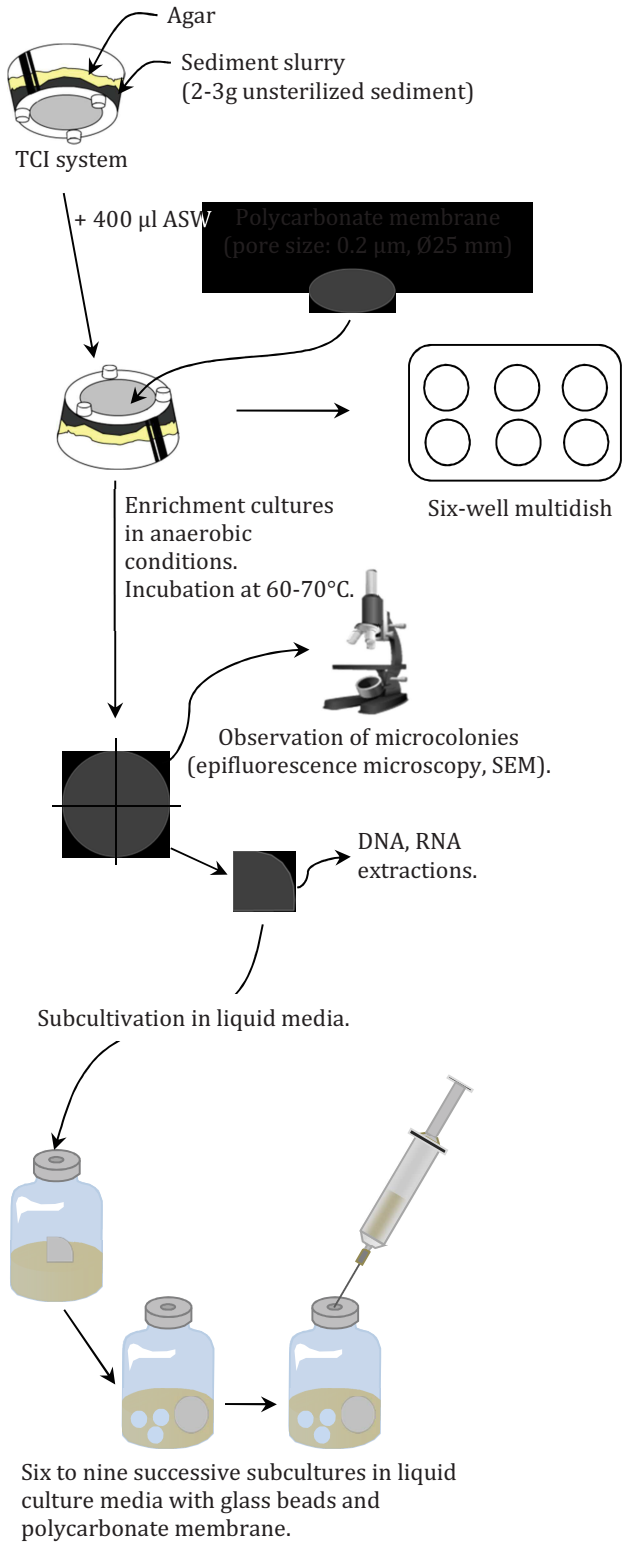

Figure S8 Culture flow-diagram.
Legend: TCI, tissue culture insert; ASW, artificial seawater.

Etude 2 : In-depth analyses of deep subsurface sediments using 454-pyrosequencing revealed a reservoir of buried fungal communities.

FEMS – Microbiology Ecology, accepté

Rédou V., Ciobanu M.C., Pachiadaki M.G., Edgcomb V., Alain K., Barbier G. and Burgaud G.

Dans une seconde étude complémentaire, je me suis intéressée plus précisément à la composante fongique par du séquençage haut-débit d'amplicons en me basant sur un marqueur génétique largement utilisé pour la description des communautés fongiques, l'ITS1 (*Internal Transcribed Spacer 1*). L'objectif de cette étude était d'obtenir une vision plus précise des communautés fongiques des sédiments profonds en utilisant deux marqueurs moléculaires, l'ARNr 18S et l'ITS1, permettant ainsi une assignation taxonomique au niveau de l'espèce. Du fait de la relative stabilité et de la préservation de l'ADN dans les sédiments, nous avons utilisé en complément une approche similaire basée sur les ARNr et ciblant la région hypervariable V4 de l'ARNr eucaryote afin de mettre en évidence la composante active des champignons dans cet écosystème.

Bien que la diversité fongique globale soit relativement faible, une diversité plus importante a été observée dans les premières couches sédimentaires, de 346 à 583 mbsf, que dans les échantillons les plus profonds. En effet, après la transition sédiments/roches sédimentaires, autour de 931 mbsf, la diversité diminue. Ces communautés fongiques pourraient dépendre de la diminution de la porosité ou de la disponibilité de la matière organique, plus faible en profondeur. Cependant, il semblerait que certaines communautés soient négativement corrélées avec la matière organique ce qui pourrait indiquer une adaptation à des conditions oligotrophes. De manière intéressante, les 22 genres fongiques mis en évidence dans cette étude sont phylogénétiquement proches de champignons terrestres,

ce qui suggère que ces champignons semblent capables de s'adapter et de coloniser des environnements marins et de persister à de grandes profondeurs dans les sédiments malgré les contraintes physico-chimiques et énergétiques *in situ*. Bien que nos jeux de données ARN et ADN ne soient pas strictement comparables, il semblerait que la majorité des champignons du bassin de Canterbury soit en dormance. De précédentes études moléculaires ont suggéré que la levure *Malassezia* semblait omniprésente dans des environnements marins extrêmes. Nos résultats apparaissent donc en cohérence avec ces études car plusieurs séquences d'ADN et d'ARNr affiliées à levure *Malassezia* ont été obtenues. Bien qu'aucun isolat de *Malassezia* n'ait encore été obtenu à partir d'environnements extrêmes, cet organisme semble avoir une importance écologique dans ces écosystèmes.

A notre connaissance, ce travail est le premier spécifiquement dédié à la description des communautés fongiques dans les sédiments de subsurface et fournit la preuve que les communautés fongiques peuvent persister à des profondeurs records dans les sédiments du bassin Canterbury. En effet, nos résultats démontrent des signatures fongiques ADN et ARNr et révèlent leur présence jusqu'à 1740 mbsf et une activité fongique à 346 mbsf.

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RESEARCH ARTICLE

In-depth analyses of deep subsurface sediments using 454-pyrosequencing reveals a reservoir of buried fungal communities at record-breaking depths

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454 pyrotag; Canterbury basin; deep subseafloor; fungi; microeukaryotes.

Abstract

The deep subseafloor, extending from a few centimeters below the sediment surface to several hundred meters into sedimentary deposits, constitutes the deep biosphere and harbors an unexpected microbial diversity. Several studies have described the occurrence, turnover, activity and function of subseafloor prokaryotes; however, subsurface eukaryotic communities still remain largely underexplored. Ribosomal RNA surveys of superficial and near-surface marine sediments have revealed an unexpected diversity of active eukaryotic communities, but knowledge of the diversity of deep subseafloor microeukaryotes is still scarce. Here, we investigated the vertical distribution of DNA and RNA fungal signatures within subseafloor sediments of the Canterbury basin (New Zealand) by 454 pyrotag sequencing of fungal genetic markers. Different shifts between the fungal classes of *Tremellomycetes*, *Sordariomycetes*, *Eurotiomycetes*, *Saccharomycetes*, *Wallemiomycetes*, *Dothideomycetes*, *Exobasidiomycetes* and *Microbotryomycetes* were observed. These data provide direct evidence that fungal communities occur at record depths in deep sediments of the Canterbury basin and extend the depth limit of fungal presence and activity, respectively 1740 and 346 mbsf. As most of the fungal sequences retrieved have a cosmopolitan distribution, it indicates that fungi are able to adapt to the deep subseafloor conditions at record-depth and must play important ecological roles in biogeochemical cycles.

Introduction

Exploration of the marine subsurface was initiated in the 1930s (ZoBell & Anderson, 1936) and since that time it has been demonstrated that abundant and diverse microbial communities inhabit this ecosystem with the potential to impact large-scale biogeochemical cycles (Fredrickson & Balkwill, 2006; Edwards *et al.*, 2012; Anderson *et al.*, 2013). The deep subseafloor biosphere hosts large numbers of living microbial cells, estimated at 2.9×10^{29} cells, as revealed by recent cell counts (Kallmeyer *et al.*, 2012) and thus represents a significant biome structured by several spatial, physical and energetic constraints (e.g. confinement, pressure, temperature, refractory organic matter, etc.). Studies of subsurface microbial communities (Parkes *et al.*, 2000; Fry *et al.*, 2008; Orcutt *et al.*, 2011) and metabolisms (D'Hondt *et al.*, 2002, 2004; Biddle *et al.*, 2006, 2011) have provided a foundation for understanding the ecological roles of subsurface microorganisms. Prokaryotes remain the common targets of investigations that aim to study subsurface microbial diversity, and debate continues whether *Archaea* or *Bacteria* predominate (Schippers *et al.*, 2005; Biddle *et al.*, 2006; Briggs *et al.*, 2012).

Microeukaryotes remain understudied although their presence in marine extreme environments is increasingly

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documented. Culture-based and culture-independent methods have demonstrated their occurrence in hydrothermal vents (Edgcomb *et al.*, 2002; Burgaud *et al.*, 2009, 2010; Le Calvez *et al.*, 2009), anoxic environments (Stoeck & Epstein, 2003; Takishita *et al.*, 2005; Jebaraj *et al.*, 2010), deep hypersaline anoxic basins (Alexander *et al.*, 2009; Stock *et al.*, 2012), cold seeps (Takishita *et al.*, 2007; Nagano *et al.*, 2010; Nagahama *et al.*, 2011) and associated with sunken wood (Barghoorn & Linder, 1944; Dupont *et al.*, 2009). The search for microeukaryotes in the deep subseafloor has been delayed by the prevailing viewpoint that spatial constraints were an obstacle to the growth of larger cells or multicellular microorganisms. However, deep systems with cavities of several microns appear large enough to support microeukaryotic life (Ciobanu *et al.*, 2014). Recent studies have definitely demonstrated microeukaryotic presence, activity and metabolisms in the marine subsurface at depths from 5 to 159 met below seafloor (mbsf; Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a, b). Among microeukaryotes, fungal communities appear not to be diverse (Edgcomb *et al.*, 2011; Xu *et al.*, 2014) but consistently dominate, and thus may have significant ecological roles in the deep biosphere. Fungi revealed in marine sediments are typically widespread in terrestrial environments (Richards *et al.*, 2012), indicating that terrestrial and surface-dwelling fungi may be capable of adaptation to deep biosphere conditions and thus may be capable of colonizing the deep subseafloor. Ciobanu *et al.* (2014) recently extended the boundaries of microbial life using a record-depth (1922 m) sediment core from the Canterbury basin (New Zealand).

That sediment core was investigated to test hypotheses regarding the limits of the deep biosphere, and revealed the occurrence of *Bacteria*, *Archaea* and *Eukarya* at these record depths in the subseafloor. In our study, we conducted an in-depth investigation of microeukaryotic communities from Canterbury basin sediment cores to provide further insights on the nature and extent of subsurface fungal reservoirs in the deep biosphere. We used a 454-pyrosequencing approach targeting eukaryotic small subunit (18S) ribosomal RNA and DNA, and fungal ITS1 regions in order to analyze the presence and activity of the different fungal operational taxonomic units (OTUs) present at the species level, a prerequisite to the understanding of the ecological roles of fungi in the deep biosphere.

Here we show that fungal signatures appear at record-depth in the deep subseafloor, although species richness is extremely low, with only 18 OTUs detected. Based on rDNA and rRNA sequences detected at 1740 and 346 mbsf, respectively, fungi may be viewed as a third microbial component (after *Bacteria* and *Archaea*) with potentially important ecological roles in the deep biosphere.

Materials and methods

Site description and sediment sampling

Sediment samples were collected from the Canterbury basin, on the eastern margin of the South Island of New Zealand, during IODP Leg 317 Expedition (RV *JOIDES Resolution*). A sediment core was drilled at Site U1352 (44°56′26.62″S, 172°1′36.30″E; Fig. 1) in 344 m water depth, and the sediment core had a depth of 1927.5 mbsf, spanning the Holocene to late Eocene periods. The core lithology and environmental parameters at the time of sampling are described in Ciobanu *et al.* (2014).

Nucleic acid extractions, PCR amplifications and 454 sequencing

DNA extractions were performed from nine samples collected along the core (Table 1). To avoid contamination, all manipulations were carried out in a PCR cabinet exclusively dedicated to low biomass sediment samples (PCR cabinet; Captair® Bio, Erlab). DNA was extracted from 5 × 0.5–1 g frozen samples (−80 °C), where no fluorescent microspheres could be retrieved, with the FastDNATM Spin Kit for Soil (#6560-200; MP Biomedicals®), following the manufacturer's instructions. Ten microliters of linear acrylamide (5 mg mL^{-1}; Ambion/Applied Biosystems) were added to the protein lysis buffer in order to favor DNA precipitation in subsequent stages. At the final step, DNA was eluted in a 50-μL volume. Concentration of extracted DNA was measured with a NanoDrop 1000 Spectrophotometer (Thermo-Scientific). Negative controls (reaction mixture without DNA or cDNA) were included in each set of PCR reactions. In addition, a negative control (e.g. negative DNA or cDNA extraction) was prepared for each work stage to ensure that no contamination with exogenous amplifiable DNA or cDNA occurred during the different stages of sample treatment.

For each DNA extract, four independent 25-μL PCR amplifications were performed using the universal eukaryotic primers Euk 42F (López-García *et al.*, 2003)/Euk 516R (Amann *et al.*, 1990) and fungal primers ITS1 (Gardes & Burns, 1993)/ITS2 (White *et al.*, 1990). Nested PCR amplifications followed with fusion primer sets Euk 82F (Dawson & Pace, 2002)/Euk 516R and ITS1F/ITS2 (White *et al.*, 1990). All PCR reactions were performed in 25-μL volumes containing 1 × Taq DNA polymerase buffer with $MgCl_2$ (2 mM), 1 mM of additional $MgCl_2$, 240 μM dNTP, 0.4 μM of each primer, 1 volume of

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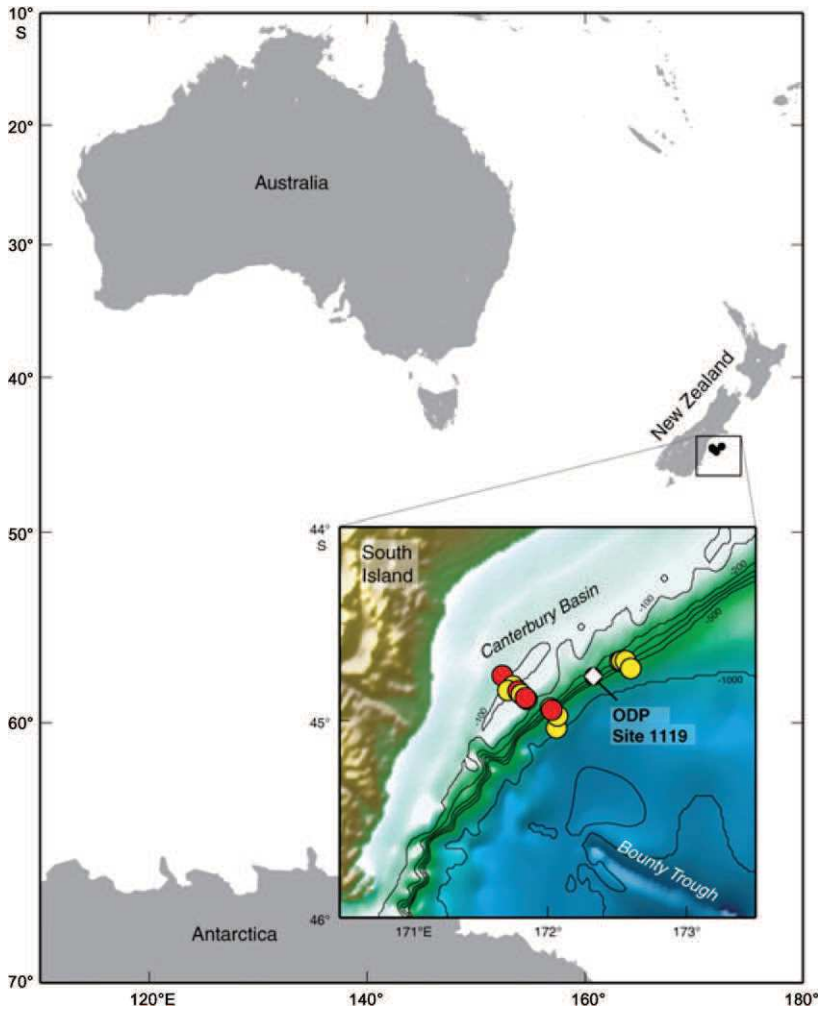

Fig. 1. Sampling site.

Table 1. List of studied samples and depths

Sample name	Core depth below the seafloor (m)	Study performed	Target marker
2H	12	cDNA	18S rDNA
42X	346	DNA/cDNA	18S rDNA/ITS
68X	583	DNA	18S rDNA/ITS
73X	634	DNA	18S rDNA
34R	931	DNA/cDNA	18S rDNA/ITS
87R	1367	DNA	18S rDNA
110R	1577	DNA	ITS
122R	1690	DNA	18S rDNA
125R	1711	DNA	ITS
128R	1740	DNA	18S rDNA

5× GC-rich buffer, 1 unit of FastStart Taq DNA polymerase, and 1 μL of DNA template. The first PCR assay for ITS1 region started by initial denaturation step at 95 °C for 5 min, followed by 20 cycles of 1 min at 95 °C, 1 min at 56 °C and 1.5 min at 72 °C and a final extension step of 7 min at 72 °C. The second PCR amplification was performed in the same conditions using 10 cycles. PCR products were pooled two by two so as to have two independent replicates for pyrosequencing. The PCR program for 18S and sequencing strategy were described in detail previously (Ciobanu *et al.*, 2014).

RNA extractions were performed using three samples collected along the core, including 12, 346 and 931 mbsf (Table 1). RNA was extracted from 2 × 4 g for the 12-mbsf sample and around 2 × 8 g for 346- and 931-mbsf samples of uncontaminated frozen samples (−80 °C) with the RNA PowerSoil® Total RNA Isolation Kit (#12866-25; MO BIO Laboratories), following the manufacturer's instructions. To purify the RNA extracts, the MEGAclear RNA purification kit (Life Technologies) was used according the manufacturer's instructions. To remove any potential DNA contamination, RNA extracts were treated

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using the Turbo DNA-free kit (Life Technologies). Removal of contaminating DNA was confirmed by the absence of visible amplification of small subunit ribosomal RNA genes using bacterial hypervariable V4 region primers (Cole *et al.*, 2009) after 40 cycles of PCR using the RNA extracts as template. Total extracted RNA was immediately reverse-transcribed to cDNA using the QuantiTect Transcription Kit (Quiagen) according the manufacturer's instructions. The universal primers TAReukFWD1 and TAReukREV3 (Stoeck *et al.*, 2010) were used to amplify the V4 region (*c.* 380 bp) of the eukaryotic 18S rRNA gene. All PCR reactions were performed in 25-μL reaction volumes containing 1× GoTaq Buffer, 0.2 mM dNTPs, 0.4 μM of each primer, 2.5 mM of $MgCl_2$, 1 U of GoTaq polymerase and 1 μL of cDNA. The PCR assay started by initial denaturation step at 95 °C for 4 min, followed by 35 cycles of 35 s at 95 °C, 45 s at 48 °C and 50 s at 72 °C and a final extension step of 7 min at 72 °C. Amplicons were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions. Barcodes were added by ligation by the sequencing company (Molecular Research LP, Texas) and pyrosequencing was performed on a GS FLX+ platform (Roche).

Quality control, clustering and taxonomic assignment of 454 pyrotags

DNA- and RNA-based 18S data processing

The raw sff files were converted to FASTQ format. A quality filtering was performed with USEARCH using the maximum expected error filtering method. Sequences for which all base pairs had a Phred quality score under 15 were removed. A minimum read length retained after trimming was set at 200 bp. The clustering was performed using USEARCH v7 (Edgar, 2010) and the UPARSE function (Edgar, 2013). The removal of duplicated sequences was performed using USEARCH v7 and the derep_fulllength command with the sizeout option set at 64. Dereplicated reads were sorted by decreasing abundance with the sortbysize command. OTUs were delineated at a 97% identity threshold using the cluster_otus command. Only OTUs present in both sequencing duplicates were retained. Chimeric sequences were identified and removed using Silva_111 (Quast *et al.*, 2013) as the reference database for eukaryotes within UCHIME (Edgar *et al.*, 2011). The OTU table generated from the UPARSE pipeline was processed and analyzed using the QIIME pipeline (Caporaso *et al.*, 2010). Taxonomic assignment was performed using the SILVA_111 database and including only eukaryotic sequences with the assign_taxonomy.py command within QIIME. The taxonomic mapping file was generated with the assign_taxonomy.py command. Sequence representatives of each OTU were aligned using PYNAST (DeSantis *et al.*, 2006) and classified using the SILVA_111 rep set. The alignment was filtered using default parameters. The 10% most variable positions within the alignment and positions that contained > 80% gaps were removed. A eukaryotic phylogenetic analysis was performed with the make_phylogeny.py command within QIIME. Both alpha and beta diversity metrics were determined using the core_diversity_analyses.py command in QIIME. Alpha diversity was assessed by calculating the richness estimator Chao 1 (Chao, 1984) and the Simpson and Shannon diversity indices (Simpson, 1949; Shannon & Weaver, 1963). Beta diversity patterns of samples were assessed using unweighted and weighted UniFrac metrics (Lozupone & Knight, 2005).

DNA-based ITS1 data processing

Data processing used for the ITS1 marker was similar to 18S data processing from raw sff files to the dereplication step. Chimeric sequences were identified and removed using UNITE (Abarenkov *et al.*, 2010) as a reference database for fungi (Kõljalg *et al.*, 2013), within UCHIME (Edgar *et al.*, 2011). OTU table generated from UPARSE pipeline were processed and analyzed using QIIME pipeline (Caporaso *et al.*, 2010). Taxonomic assignment was performed using UNITE database with assign_taxonomy.py command. Taxonomic mapping file was performed with the assign_taxonomy.py command. Both alpha and beta diversity metrics were determined using the core_diversity_analyses.py command in QIIME. Alpha diversity was assessed by calculating the richness estimator Chao 1 (Chao, 1984) and the Simpson and Shannon diversity indices (Simpson, 1949; Shannon & Weaver, 1949). Beta diversity patterns of samples were assessed using unweighted and weighted UniFrac metrics (Lozupone & Knight, 2005).

Statistical analyses

Multiple factorial analyses (MFA) and principal component analyses (PCA) were processed to elucidate relationships between fungal community structure and some selected environmental parameters acquired during IODP Expedition 317 (Fulthorpe *et al.*, 2011). MFA allows the mapping of sediment samples on a two-dimensional plane showing the contribution of the different parameters (OTUs, Depth, Organic Carbon, Inorganic Carbon, Porosity, Methane, Ethane and Depth). PCA analyses are based on different OTUs obtained and environmental parameters for each sample. All statistical analyses were performed using XLSTAT (Addinsoft, USA, New York, NY).

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Results

Fungal diversity

DNA-based eukaryotic gene dataset based on V1–V3 region of 18S rRNA gene

A total of 28 868 sequences were generated by the 454 pyrosequencing for the nine depths from 346 to 1740 mbsf. After quality control, 48% of the sequences were analyzed (14 036 sequences) and were grouped into 13 fungal OTUs. For each sample, the total number of fungal 454 reads, the number of cleaned fungal 454 reads, and the number of fungal OTUs are summarized in Table 2. Fungi in this DNA-based 18S dataset represent 54–100% of all sequences recovered. Chao richness estimators (Table 3) are consistent with the numbers of observed OTUs, indicating a complete coverage of the fungal DNA diversity. The Shannon diversity index computed for every depth ranged from 0 to 2.11 (Table 3), suggesting a low species diversity of fungal communities along the core.

RNA-based eukaryotic gene dataset based on V4 region of 18S rRNA gene

A total of 164 743 sequences were generated for RNA-based 454 pyrotags for three depths, 12, 346 and 931 mbsf. After quality control, 39% of sequences were analyzed (63 564 sequences) and grouped into 185 OTUs, among them only one fungal OTU was observed at 12 and 346 mbsf.

DNA-based eukaryotic gene dataset based on fungal ITS1 region

A total of 17 672 sequences were generated by 454 pyrosequencing for five samples from 346 to 1711 mbsf. After quality control, 59% of ITS1 sequences were analyzed (10 421 sequences) and after removal of singletons were grouped into 18 OTUs. For each sample, the total number of fungal 454 reads obtained, the number of reads retained after quality control, and the resulting number of OTUs are summarized in Table 2. Chao richness estimators are consistent with the numbers of observed OTUs, indicating a complete coverage of the fungal DNA diversity. The Shannon diversity index computed for every depth ranged from 0 to 1.86, suggesting, as for the DNA-based 18S dataset, that the fungal communities using ITS1 are also weakly diversified along the core.

Distribution patterns of fungal communities

Variations in the structure of the fungal communities were determined MFA coupled with a PCA (Figs 2 and 3). MFA represents a convenient tool for comparing several samples characterized by the same subset of factors. Using MFA, it was possible to get an overall picture of the common structure emerging from the dataset. Differences between samples were deduced based on the superimposed representations (Figs 2a and 3a) on which samples are represented as centers of gravity of the different variables. MFA clearly positioned depth and porosity as strong structuring parameters with low depth/high porosity samples on the right of the plot and high depth/low porosity samples on the left (Figs 2a and 3a). It also clearly highlighting the complexity of the diversity with complex vs. not complex samples, as detailed below. MFA allowed us to infer general distribution patterns. Then, PCA was then used to detail community distribution.

DNA-based 18S dataset

The MFA representation allowed samples with close values to be clustered, i.e. 1367-, 634- and 1740-mbsf samples for

Table 2. Number of sequences and number of OTUs in the DNA-based datasets

	346 mbsf		583 mbsf		634 mbsf	931 mbsf		1367 mbsf	1577 mbsf	1690 mbsf	1711 mbsf	1740 mbsf
	18S	ITS	18S	ITS	18S	18S	ITS	18S	ITS	18S	ITS	18S
Number of reads	6425	4625	5868	3789	7646	4521	1485	2318	4377	2866	3399	2659
Retained quality reads	2491	2366	4747	1448	2973	2260	983	919	3568	1809	2056	1240
Number of fungal OTUs	7	3	7	10	2	4	6	1	1	3	2	3

Table 3. Diversity indices for the 18S surveys calculated based on the fungal OTUs

	346 mbsf		583 mbsf		634 mbsf	931 mbsf		1367 mbsf	1577 mbsf	1690 mbsf	1711 mbsf	1740 mbsf
	18S	ITS	18S	ITS	18S	18S	ITS	18S	ITS	18S	ITS	18S
Richness (Chao1)	7	4	10	11	2	4	6	1	1	3	2	3
Diversity (Shannon)	2.11	1.04	1.21	1.71	0.43	0.36	1.86	0	0	1.28	0.07	1.44
Evenness (Simpson)	0.73	0.42	0.51	0.47	0.16	0.12	0.64	0	0	0.54	0.02	0.60

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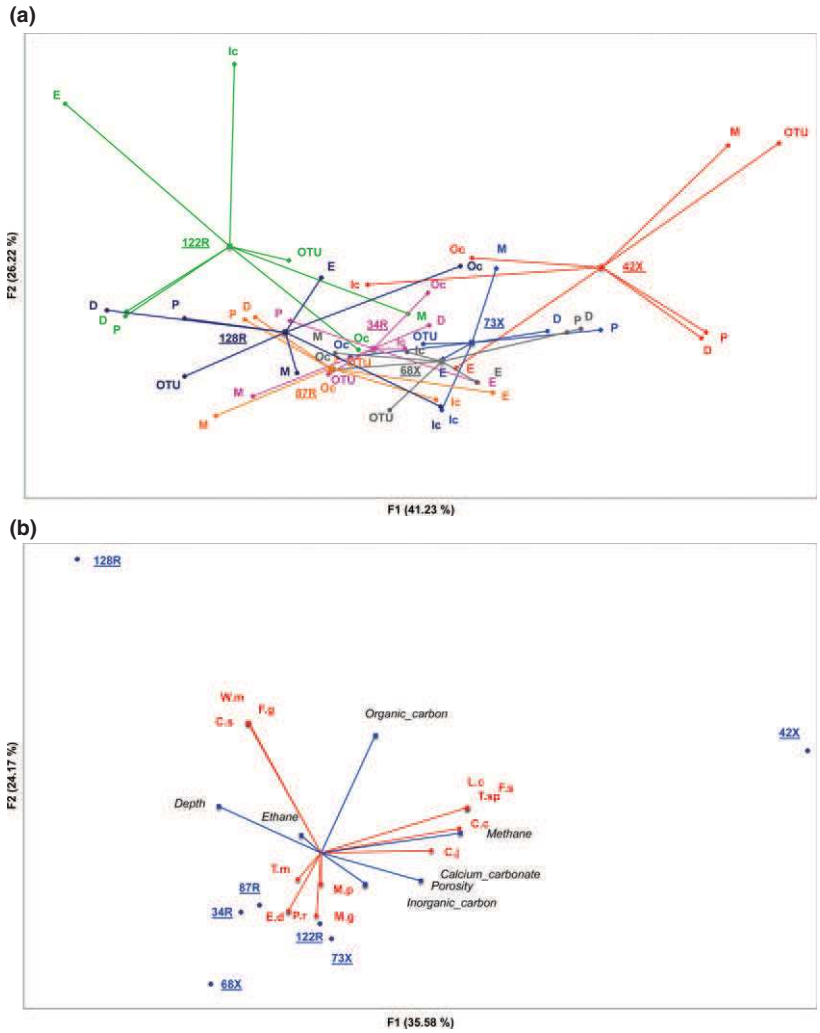

Fig. 2. MFA and PCA based on 454-pyrosequencing of 18S reads and environmental parameters. (a) Superimposed representation individuals (sediment samples) with the contribution of OTUs and the different environmental parameters. D, depth; E, ethane; Ic, inorganic carbon; M, methane; Oc, organic carbon; P, porosity. (b) Distribution of sediment samples, OTUs and environmental parameters. C.c, *Cryptococcus curvatus*; C.j, *Cyberlindnera jadinii*; C.s, *Cryptococcus surugaensis*; E.d, *Exophiala dermatitidis*; F.g, *Filobasidium globisporum*; F.s, *Fusarium solani*; L.c, *Leptosphaerulina chartarum*; M.g, *Meyerozyma guilliermondii*; M.p, *Malassezia pachydermatis*; P.r, *Pleurostomophora richardsiae*; T.m, *Trichosporon mucoides*; T.sp, *Trichoderma* sp.; W.m, *Wallemia muriae*. Sample depths are given in Table 1.

inorganic carbon and 346-, 1740- and 931-mbsf samples for organic carbon, as well as differentiating some samples, for example, 583-, 634- and 1367-mbsf samples were inversely correlated with 346-, 931- and 1740-mbsf samples for organic carbon. MFA clearly indicates that 346-, 931- and 1740-mbsf samples represented a relatively organic-rich depth compared with 583-, 634- and 1367-mbsf samples. MFA allowed differentiation of sediment samples based on diversity, that is, complex or not complex. The 346- and 583-mbsf samples, with long-length OTU vectors, represented complex samples compared with the other sediment samples with short-length OTU vectors, demonstrating that fungal diversity was higher in the first sediment layers analyzed. Using the OTU vector, MFA also clearly indicated a strong diversity at the different depths. The lengths and also the different OTU vector directions at different depths confirm that the first sediment layers analyzed were more complex and diverse. PCA allowed visualization of three clusters: (I) *Wallemia muriae*, *Filobasidium globisporum*, *Cryptococcus surugaensis*; (II) *Trichosporon mucoides*, *Malassezia pachydermatis*, *Meyerozyma guilliermondii*, *Pleurostomophora richardsiae*, *Exophiala dermatitidis*, and (III) *Leptosphaerulina chartarum*, *Fusarium solani*, *Trichoderma* sp., *Cryptococcus curvatus* and *Cyberlindnera jadinii*. The first cluster was mainly composed of OTUs from the deeper sediment horizon

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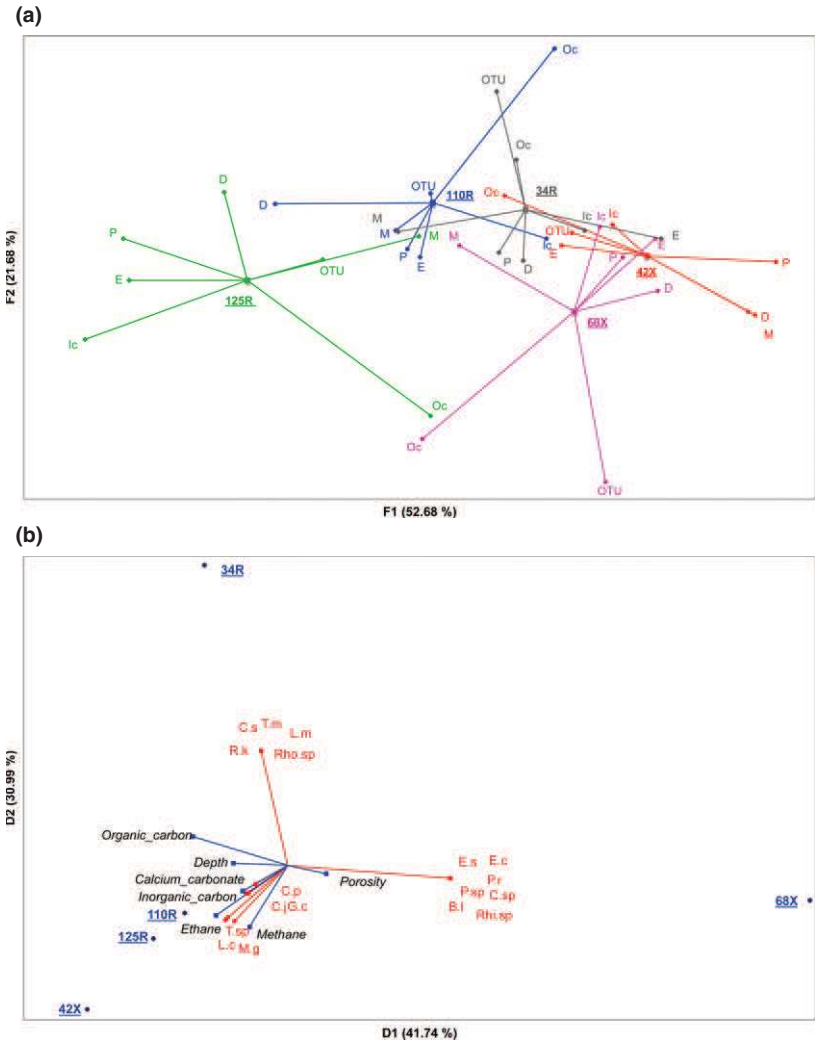

Fig. 3. MFA and PCA based on 454-pyrosequencing of ITS reads and environmental parameters. (a) Superimposed representation individuals (sediment samples) with the contribution of OTUs and the different environmental parameters. D, depth; E, ethane; Ic, inorganic carbon; M, methane; Oc, organic carbon; P, porosity. (b) Distribution of sediment samples, OTUs and environmental parameters. B.l, *Batcheloromyces leucadendri*; C.j, *Cyberlindnera jadinii*; C.p, *Cryptococcus pseudolongus*; C.s, *Cryptococcus saitoi*; C.sp, *Chaetothyriales* sp.; E.c, *Elmerina caryae*; E.s, *Exophiala spinifera*; G.c, *Galactomyces candidum*; L.c, *Leptosphaerulina chartarum*; L.m, *Leucosporidiella muscorum*; M.g, *Meyerozyma guilliermondii*; P.r, *Pleurostomophora richardsiae*; P.sp, *Penicillium* sp.; R.k, *Rhodosporidium kratochvilovae*; Rhi.sp, *Rhinocladiella* sp.; Rho.sp, *Rhodotorula* sp.; T.m, *Tremella moriformis*; T.sp, *Trichosporon* sp. Sample depths are given in Table 1.

depth, whereas cluster III contained only OTUs from the shallowest depth. In cluster III, *C. curvatus* appeared significantly correlated with methane. Cluster II appeared negatively correlated with organic carbon, indicating that *T. mucoides*, *M. pachydermatis*, *E. dermatitidis*, *P. richardsiae* and *M. guilliermondii* were OTUs only found in sediment samples with a low organic carbon concentration.

DNA-based ITS1 dataset

Consistent with the 18S data, MFA indicated that 346-, 583- and 931-mbsf samples, with long-length OTU vectors, were more complex than 1577- and 1711-mbsf samples. As found for 18S data, the different OTU vector directions at the different depths clearly indicate contrasted fungal communities along the sediment core. PCA clearly differentiated three clusters: (I) *Cryptococcus saitoi*, *Rhodosporidium kratochvilovae*, *Rhodotorula* sp., *Tremella moriformis*, *Leucosporidiella muscorum*; (II) *P. richardsiae*, *Chaetothyriales* sp., *Exophiala spinifera*, *Penicillium* sp., *Batcheloromyces leucadendri*, *Elmerina caryae*, *Rhinocladiella* sp., and (III) *Cryptococcus pseudolongus*, *C. jadinii*, *Galactomyces candidum*, *L. chartarum*, *Trichosporon* sp., and *M. guilliermondii*. Those three clusters appeared depth-

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specific, and most of the OTUs were only found at a given depth. Many OTUs belonging to clusters II and III on the right of the PCA were inversely correlated with organic matter. Members of the cluster III, some of which were affiliated to the *Crypococcus* genus, appeared to be correlated with methane and ethane.

Of the 13 OTUs obtained with eukaryotic primers, six appear to be depth-dependent: OTU 2, OTU 3, OTU 6, OTU 9, OTU 10, OTU and OTU 13 (Table 4). A maximum of four OTUs are shared between samples, observed in the same sample type: OTU 4, OTU 5, OTU 8 and OTU 11. In the ITS1 dataset, the 346-mbsf sample shared OTU 3 and OTU 6 with the 583-mbsf sample and OTU 3 with the 931-mbsf sample. The 583-mbsf sample shared OTU 3 with the 931-mbsf sample and OTU 2 with the 1711-mbsf sample (Table 4).

Taxonomic composition

To evaluate the taxonomic composition of each sample, the representative reads were compared against SILVA_111, UNITE and GenBank databases.

Table 4. List of fungal operational taxonomic units found in deep-sea sediment using eukaryotic V1–V3 primers (a) and fungal ITS1 primers (b)

OTU Id	Sample depth (mbsf)	Most similar sequence	*E* value	Identity (%)	GenBank accession number of the most similar sequence
(a)					
OTU_1	583, 634, 931, 1367, 1690	*Meyerozyma guilliermondii*	2.00E-131	261/262 (99)	JQ698913.1
OTU_2	931	*Trichosporon mucoides*	3.00E-130	259/260 (99)	AB001763.2
OTU_3	583	*Pleurostomophora richardsiae*	7.00E-176	338/338 (100)	AY729812.1
OTU_4	346, 583, 931, 1690	*Cryptococcus curvatus*	2.00E-131	261/262 (99)	AB032626.1
OTU_5	346, 583, 1740	*Cryptococcus surugaensis*	0.0	360/361 (99)	AB100440.1
OTU_6	346	*Leptosphaerulina chartarum*	3.00E-174	338/339 (99)	HM216185.1
OTU_7	346, 1740	*Filobasidium globisporum*	2.00E-131	261/262 (99)	AB075546.1
OTU_8	346, 583, 634	*Cyberlindnera jadinii*	3.00E-174	338/339 (99)	EF550447.1
OTU_9	583	*Exophiala dermatitidis*	4.00E-129	257/258 (99)	X79317.1
OTU_10	1740	*Wallemia muriae*	1.00E-124	246/246 (100)	AY741381.1
OTU_11	346, 583	*Trichoderma* sp.	2.00E-176	339/339 (100)	AB491674.1
OTU_12	346, 1690	*Malassezia pachydermatis*	2.00E-147	327/345 (95)	DQ457640.1
OTU_13	346	*Fusarium solani*	5.00E-118	237/238 (99)	AB473810.1
(b)					
OTU_1	1577	*Cryptococcus pseudolongus*	3.00E-55	121/121 (100)	AB051048.1
OTU_2	583, 1711	*Cyberlindnera jadinii*	4.00E-98	201/202 (99)	FJ865435.1
OTU_3	346, 583, 931	*Trichosporon* sp.	9.00E-100	204/205 (99)	JX270559.1
OTU_4	583	*Pleurostomophora richardsiae*	3.00E-44	101/101 (100)	KF751183.1
OTU_5	931	*Cryptococcus saitoi*	1.00E-133	262/262 (100)	JX188127.1
OTU_6	346, 583	*Meyerozyma guilliermondii*	6.00E-136	266/266 (100)	KC119207.1
OTU_7	931	*Rhodosporidium kratochvilovae*	1.00E-92	188/188 (100)	JN662395.1
OTU_8	346	*Leptosphaerulina chartarum*	3.00E-104	214/216 (99)	AM231400.1
OTU_9	583	*Exophiala spinifera*	9.00E-50	111/111 (100)	JX966556.1
OTU_10	583	*Penicillium* sp.	2.00E-86	177/177 (100)	KC464351.1
OTU_11	931	*Leucosporidiella muscorum*	7.00E-86	187/192 (97)	FR717869.1
OTU_12	931	*Rhodotorula* sp.	4.00E-40	106/112 (95)	AM901696.1
OTU_13	583	*Chaetothyriales* sp.	3.00E-35	153/183 (84)	HQ634635.1
OTU_14	583	*Elmerina caryae*	3.00E-24	125/152 (82)	JQ764655.1
OTU_15	583	*Rhinocladiella* sp.	3.00E-59	128/128 (100)	KF811431.1
OTU_16	931	*Tremella moriformis*	3.00E-60	132/133 (99)	AF042426.1
OTU_17	1711	*Galactomyces candidum*	4.00E-73	161/164 (98)	JN974290.1
OTU_18	583	*Batcheloromyces leucadendri*	7.00E-46	122/131 (93)	JF499832.1

OTU Id	Sample depth (mbsf)	Most similar sequence	*E* value	Identity (%)	UNITE accession number of the most similar sequence
OTU_10	583	*Penicillium minioluteum*	7.00E-89	100	SH225847.06FU
OTU_12	931	*Rhodotorula minuta*	2.00E-47	97	SH227573.06FU
OTU_15	583	*Rhinocladiella similis*	8.00E-62	100	SH210380.06FU

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DNA-based eukaryotic 18S dataset

Within the fungal kingdom, only the *Dikarya* were detected in our DNA-based libraries, with 63% of *Ascomycota* and 37% of *Basidiomycota*. The reads were classified into seven classes. Signatures of *Saccharomycetes* and *Tremellomycetes* were the most abundant recovered, with 38% and 35% of the reads, respectively. *Sordariomycetes*, *Eurotiomycetes*, *Dothideomycetes*, *Wallemiomycetes* and *Exobasidiomycetes* were less well represented, with 15%, 6%, 3%, 2% and 1% of the reads, respectively. Signatures of *Saccharomycetes* and *Tremellomycetes* had a broad distribution pattern, with no apparent specificity for one depth. *Dothideomycetes*, *Sordariomycetes* and *Eurotiomycetes* were only detected in the first samples from 346 to 583 mbsf. Sequences affiliated to *Wallemiomycetes* were seen exclusively in our deepest sample from 1740 mbsf. OTUs with a species-level taxonomic assignment are presented in Table 4. Fungal species appear to be unique at the different depths analyzed. *Fusarium solani* and *L. chartarum* were only found in the 346-mbsf sample. *Cyberlindnera jadinii*, *E. dermatitidis*, *Trichoderma* sp. and *P. richardsiae* were detected in the shallowest samples up to 634 mbsf. Signatures of *T. mucoides* were only detected in the 931-mbsf sample, whereas *M. guilliermondii*, *C. curvatus* and *C. surugaensis* were detected in all samples analyzed. *Malassezia pachydermatis* was only detected at 931–1690 mbsf. *Filobasidium globisporum* and *W. muriae* were only found in the deepest layer (Fig. 4).

V4 region of the RNA-based eukaryotic 18S rRNA dataset

Within the fungal kingdom, only one fungal OTU was detected in the samples analyzed at 12, 346 and 931 mbsf using an RNA-based approach, and this was assigned to the basidiomycete yeast *Malassezia*. Occurrence of reads decreased with depth, with 11 reads (of 27 988 eukaryotic reads that passed quality control) observed at 12 mbsf and one read (of 18 897 eukaryotic reads) at 346 mbsf. No fungal signatures were detected in the 931-mbsf sample (of 17 397 eukaryotic reads).

DNA-based fungal ITS1 dataset

Sequences recovered in the ITS1 dataset were affiliated to the *Dikarya*. Dominance of *Basidiomycota* was observed with 61% of the sequences, while *Ascomycota* communities

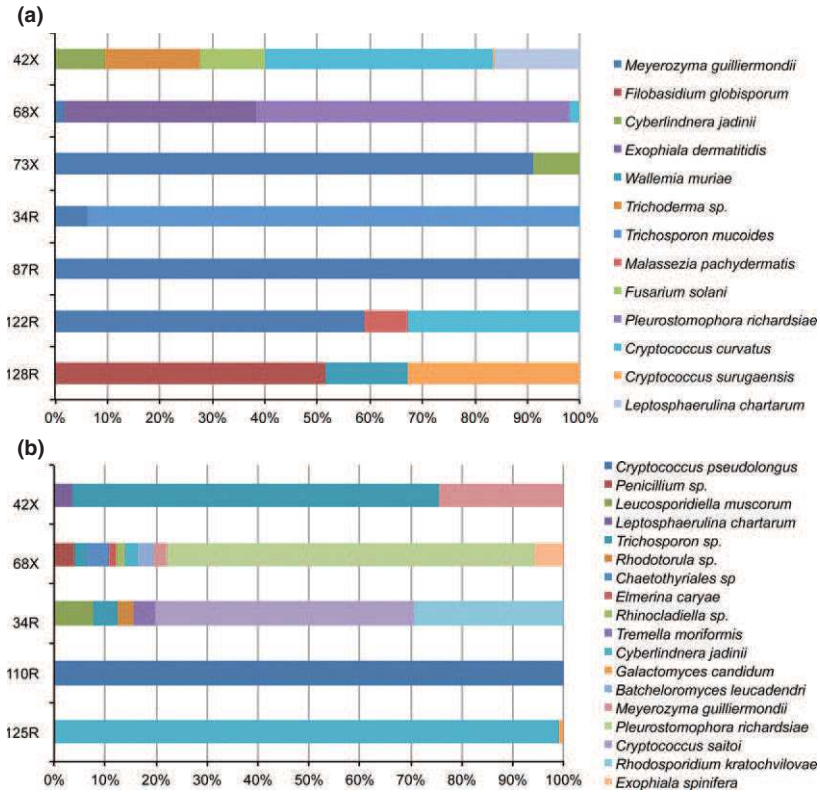

Fig. 4. Distribution of fungal OTUs at the species level based on DNA-based V1–V3 SSU (a) and ITS1 region (b). Sample depths are given in Table 1.

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were less well represented with 39% of the sequences. *Wallemiomycetes* and *Exobasidiomycetes*, which were previously found in the DNA-based 18S dataset, were absent in the ITS dataset. In contrast, *Microbotryomycetes* were only found with ITS1 primers. Signatures of *Tremellomycetes* and *Saccharomycetes* were the best represented at the class level with 57% and 26% of the reads, respectively. *Sordariomycetes*, *Microbotryomycetes*, *Eurotiomycetes* and *Dothideomycetes* represented 10%, 4%, 2% and 1% of the reads, respectively. The *Saccharomycetes* and *Tremellomycetes* displayed a broad distribution pattern, with no apparent specificity for one depth. *Dothideomycetes*, *Sordariomycetes* and *Eurotiomycetes* have been detected in the upper samples down to 582 mbsf, a result consistent with the DNA-based 18S data. OTUs and a species-level taxonomic assignment are given in Table 4. Signatures of *Penicillium* sp., *L. chartarum*, *Chaetothyriales* sp., *E. caryae*, *Rhinocladiella* sp., *B. leucadendri*, *M. guilliermondii*, *P. richardsiae* and *E. spinifera* dominated the upper horizons down to 582 mbsf. *Trichosporon* sp. was detected down to 931 mbsf. Sequences affiliating with *Leucosporidiella muscorum*, *Rhodotorula* sp., *T. moriformis*, *C. saitoi* and *R. kratochvilovae* were only recovered from the 931-mbsf sample, while *C. pseudolongus* and *C. jadinii* appeared to dominate the deepest layers analyzed (Fig. 4).

Discussion

The aim of this study was to investigate the vertical distribution of fungal communities occurring in deep subsurface sediments of the Canterbury basin. Toward that aim, we used a DNA-based analysis of eukaryotic rRNA and ITS1 signatures in samples from nine different depths from subsurface sediments collected in the Canterbury basin spanning 346 to 1740 mbsf. In addition, in a separate study included here, we applied an RNA-based analysis of rRNA genes from two depths along this transect and one complementary shallow layer sampled at 12 mbsf. These datasets provide insights into the different fungal taxa colonizing the subseafloor. The entire dataset was submitted to the European Nucleotide Archive under the study accession number PRJEB6764.

Controlling contamination

Accurate studies of the deep biosphere require strict quality controls of the samples analyzed regarding contamination risks, since the presence of exogenous cells or nucleic acids may lead to erroneous results. During the IODP 317 Expedition, the potential for microbiological contamination of samples was investigated. Different tests during drilling were performed onboard the R/V *Joides Resolution* to quantify fluorescent microbeads mimicking microbial cells that were incorporated into drill fluids during drilling. The exteriors of cores were subsequently investigated using fluorescence microscopy to determine the potential for contamination from seawater and drilling fluids. Onboard, whole-round sediment cores were subsampled from within the core interior, under sterile conditions, and these subsamples were immediately frozen at −80 °C for onshore molecular analyses. Potential contamination of the interior of the core sample is very low and was estimated at 5–11 cells g^{-1} of sediment, based on observation of fluorescent beads and average densities of 1.85 g cm^{-3} in sediments and 1.99 g cm^{-3} in sedimentary rocks at site U1352 (Ciobanu *et al.*, 2014). In addition, precautions were taken during analysis in the laboratory to avoid contamination with the use of (i) sterile materials dedicated to low biomass samples, (ii) as stringent a data processing strategy as possible (two independent pyrosequencing replicates) and (iii) the analysis of a contaminant library to remove any potential contaminant and to present a conservative picture of subsurface communities.

Comparison of the DNA-based V1–V3 SSU and ITS1 markers for understanding fungal community diversity

Among the DNA-based 18S and fungal ITS1 datasets, only the *Dikarya* were detected, suggesting absence or really low abundance of early diverging lineages in these samples. The ratio of *Ascomycota* to *Basidiomycota* was quite different, with 1.7 of the total reads using universal eukaryotic primers and 0.64 of the total reads using the fungal specific primers. However, high numbers of OTUs belonging to *Basidiomycota* in the ITS dataset are consistent with previous studies where basidiomycete yeasts were found to be the dominant fungal forms in deep-sea environments (Takishita *et al.*, 2006; Bass *et al.*, 2007; Singh *et al.*, 2011). Signatures of many common genera were detected in the two datasets – *Cryptococcus*, *Meyerozyma*, *Exophiala*, *Trichosporon*, *Pleurostomophora* and *Leptosphaerulina* – which showed the same distribution pattern along the core. *Leptosphaerulina* was found only at 346 mbsf. Similarly, *Pleurostomophora* and *Exophiala* were only detected at 583 mbsf. These genera, associated with the lower depths, seem to be correlated with high porosity and high organic carbon concentration. Sequences affiliated to *M. guillermondii* formed the most abundant OTU in the eukaryotic 18S dataset and signatures of this organism were present throughout the sediment core. By contrast, signatures of this taxon were only present in the upper sedimentary layers with the fungal ITS1 marker. *Cryptococcus* was detected all along the core with the eukaryotic 18S marker, but only in the deeper layers with the fungal marker. These differences between datasets were not

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surprising, since biases of primers are well known and each primer set favors recovery of specific taxonomic groups of fungi. Also, although ITS1 and ITS2 share many properties, the ITS2 marker is less variable in length compared with ITS1 and is also well represented in databases (Lindahl *et al.*, 2013). Multiple-primer and multiple-marker approaches thus appeared more efficient for capturing a broader picture of fungal diversity.

Fungal communities in deep sediments are not diverse and are close to terrestrial taxa

Using the tag-encoded 454 pyrosequencing approach, we discovered that the fungal diversity is quite low in these samples. Fungal communities appear to be different at different depths since unique OTUs were detected at each sediment depth. Our study identified 22 different genera among the *Dikarya*. Interestingly, these deep sediment fungi are phylogenetically close to known terrestrial fungi, suggesting that fungi are able to colonize deep-sea habitats. Indeed, it has been shown that the fungi are able to change their membrane composition to tolerate *in situ* conditions such as a high hydrostatic pressure (Simonato *et al.*, 2006). One of the most abundant OTUs in our dataset was affiliated to *Cryptococcus.* This is consistent with another study of microeukaryotic diversity in deep-sea methane-rich sediments. Takishita *et al.* (2006) identified *C. curvatus* as the dominant eukaryote in 18S rRNA gene libraries from Kuroshima Knoll methane seep. This genus was also detected in subsurface sediments down to 48 mbsf in an RNA-based study of Peru Margin sediments (Orsi *et al.*, 2013a). *Cyberlindnera* yeasts were found in our study down to 1711 mbsf. These yeasts have already been shown to have the ability to colonize marine environments, including acidic waters (Gadanho & Sampaio, 2006) and subsurface sediments (Orsi *et al.*, 2013a). Other yeasts, including *Meyerozyma*, *Malassezia*, *Rhodosporidium*, *Trichosporon* and *Filobasidium* have previously been detected in marine sediments (Kutty & Philp, 2008; Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a). *Fusarium*, present down to 346 mbsf in our samples, is a genus that includes plant pathogens and mycotoxin producers. It has been reported in river and seawater (Palmero *et al.*, 2009) and it may also be an opportunistic pathogen of deep-sea animals (Ramaiah, 2006). This suggests that fungi known in terrestrial environments may also persist at greater sediment depths and might have an ecological role in the deep biosphere.

Distribution patterns

Although fungal communities exhibited quite low overall phylogenetic diversity, diversity was greater in the samples from 346 and 583 mbsf than in deeper samples. After the transition from sediment to sedimentary rock, which occurs around 931 mbsf, diversity appears to decrease. Fungal communities likely depend on a greater amount of organic material than is available in rocky subsurface horizons. Indeed, PCA of OTU distribution supports the correlation with depth and organic matter, with some communities negatively correlated with organic matter that may indicate an adaptation to oligotrophic conditions. This distribution pattern was observed with ITS1 and 18S markers. Also, a positive correlation with representatives of the *Cryptococcus* genus and methane has been revealed and supports the idea that such basidiomycetes appear to be dominant in deep-sea marine methane-rich environments (Takishita *et al.*, 2006) and that they might be indirectly involved in the deep subseafloor methane cycle. A correlation with ethane has also been revealed with *Cryptococcus* and might indicate some interactions between this fungus and methanogenic/ethanogenic prokaryotes in deep marine sediments (Hinrichs *et al.*, 2006). The poor overlap of fungal OTUs between the different depths suggests a spatial differentiation of fungal communities according to available resources at different depths, but also supports the observation that contamination is unlikely. Fungal communities appear thus mostly depth-specific with complementary environmental parameters – here organic matter and methane concentration – as structuring parameters likely to influence the distribution of the microeukaryotic communities in marine sediments.

Many fungal OTUs may represent dormant taxa

Surprisingly, only one fungal OTU was detected in our RNA-based 18S dataset compared with 13 fungal OTUs in our DNA-based 18S dataset. As a first conclusion, fungi do not appear to be very diverse or active in the Canterbury basin, in contrast to Peru Margin sediments (Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a, b). Since the RNA-based analyses were an unanticipated addition to our study, and given primers and protocols used for the RNA- and DNA-based analyses were different, variations in recovery of particular fungal OTUs must be interpreted with caution. Fungal OTUs revealed from both DNA- and RNA-based methods affiliated exclusively with the ubiquitous yeast *Malassezia.* The most ubiquitous species within the class *Exobasidiomycetes* are related to the genus *Malassezia* in deep-sea environments (Nagano & Nagahama, 2012). Although *Malassezia* species are well known as causative agents of skin diseases, this yeast is also frequently recovered by DNA-based analysis of marine samples, indicating that this taxon likely occupies a wider range of niches than previously thought, and that

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Malassezia may be common in marine environments that include deep-sea water columns and sediment samples (Amend, 2014). Gao *et al.* (2008) revealed a high diversity of *Malassezia* lineages associated with marine sponges and invertebrates, suggesting that they may also be opportunistic pathogens of deep-sea mammals. Their recovery in RNA-based clone libraries suggests that they may also survive on buried organic matter at greater sediment depths (Edgcomb *et al.*, 2011). Although molecular studies suggest the ubiquitous presence of *Malassezia* phylotypes in deep-sea environments, no cultures have been obtained from deep-sea water and deep-sea sediments. Future studies employing culture-dependent and culture-independent approaches should reveal useful information on the ecological significance of the *Malassezia* group in marine environment.

Although our RNA- and DNA-based 18S datasets are not strictly comparable, the significantly lower recovery of taxonomic diversity in the RNA-based datasets suggests that the V4 primers could be less complementary to fungal targets. The majority of subsurface fungi in this Canterbury basin subsurface sediments appear slightly active and few persistent fungal taxa seem to colonize the deep subsurface.

Conclusion

To the best of our knowledge, this work is the first dedicated to the specific description of fungal communities in the deep subseafloor. We found an unexpected fungal diversity down to a record depth of 1740 mbsf using DNA-based pyrotag sequencing. RNA provided evidence of active fungi down to 346 mbsf. The fact that some of the fungal sequences obtained in this work have been previously reported from marine ecosystems supports the hypothesis that fungal communities have an important ecological role in this ecosystem. The deep subseafloor fungi revealed in the Canterbury basin are known to be widespread in terrestrial environments, indicating that fungi are highly adaptable organisms, potentially able to colonize and have an ecological role in the deep subseafloor.

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Etude 3 : Marine Fungi from Deep Subseafloor Sediments: Species richness, Adaptation and Biotechnological potential.

Applied and Environmental Microbiology, soumis

Rédou V., Navarri M., Meslet-Cladière L., Barbier G. and Burgaud G.

Jusqu'à présent, les approches moléculaires ont mis en évidence quelques séquences d'organismes potentiellement nouveaux mais aussi de nombreuses séquences fongiques ubiquistes. Dans l'étude précédente, nous avons vu que des signatures fongiques étaient présentes jusqu'à 1740 mètres sous la surface du plancher océanique du bassin de Canterbury. Il est donc maintenant intéressant de savoir si les organismes représentés par ces séquences persistent sous forme de spores végétatives ou bien s'ils sont spécifiquement adaptés aux conditions *in situ*. Ainsi, l'isolement des représentants fongiques de la biosphère profonde est un moyen informatif d'accéder aux caractéristiques physiologiques développées par ces organismes pour s'adapter aux conditions extrêmes inhérentes à la subsurface.

Dans ce contexte, l'objectif de cette étude était de constituer pour la première fois une collection de cultures fongiques issues de sédiments marins profonds et d'évaluer leur capacité à se développer dans des conditions de pression hydrostatique élevée et de forte salinité. Dans un deuxième temps, et dans le cadre d'une approche appliquée, une recherche de gènes impliqués dans la synthèse de métabolites secondaires (enzymes, antibiotiques, acides organiques, peptides…) a été conduite afin d'évaluer le potentiel biotechnologique de la collection obtenue.

Nos résultats indiquent que des représentants des communautés fongiques de sédiments profonds peuvent être isolés en utilisant des techniques reproduisant les conditions *in situ.* Notre collection est constituée de 183 isolats fongiques principalement caractérisés comme des champignons ubiquistes. En accord avec notre précédente étude moléculaire, les

champignons semblent être en mesure de persister mais aussi de s'adapter au milieu sédimentaire profond. En effet, 80% de nos souches ont été isolées avec un enrichissement préliminaire sous pression hydrostatique élevée. Ainsi, la pression hydrostatique ne semble pas être un paramètre limitant la germination et la croissance de champignons issus de la subsurface. De plus, les souches isolées dans les couches sédimentaires les plus profondes semblent être halophiles dans des conditions de températures élevées, *i.e.* proches des températures *in situ*, et semblent indiquer un phénomène d'adaptation aux conditions environnementales.

Par leur diversité génétique et leur potentiel de synthèse de métabolites secondaires potentiellement intéressants, certains isolats de notre collection semblent représenter une ressource à exploiter dans la perspective d'applications biotechnologiques.

Applied and Environmental Microbiology

Marine Fungi from Deep Subseafloor Sediments: Species richness, Adaptation and Biotechnological potential.

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Running title:

Culturable fungal diversity of deep subseafloor sediments

Abstract

The entire fungal kingdom has unique adaptation capacities allowing them to colonize different kind of habitats, whether marine, aquatic or terrestrial. Fungi have been shown to have important ecological roles in the marine environment, from photosynthesis- to chemosynthesis-based ecosystems. Marine fungi have recently been highlighted in deep subsurface sediments using molecular methods. Those revealed microorganisms may be specifically adapted to the deep biosphere, but this can only be demonstrated using culture-based analyses. In this study, we investigated the diversity of culturable fungal communities from a sediment core collected in the Canterbury basin (New-Zealand) with the idea to assess whether they (i) are endemic species and or (ii) play active role(s). Around 200 filamentous fungi (68%) and yeasts (32%) were isolated along the sediment core. Fungi isolated were affiliated to *Ascomycota* and *Basidiomycota* phyla with 21 genera identified. Screening for genes involved in secondary metabolites pathways revealed the potential of strains isolated for synthesis of bioactive compounds. Our results provide evidence that deep sediments represent another ecological niche for fungal communities with the capacity to survive, adapt, grow and synthesize potentially useful bioactive compounds in the deep biosphere, definitely representing an untapped reservoir of microorganisms and novel sources of bioactive compounds.

Keywords

Marine, Deep sediments, Fungi, Cultures, Hydrostatic pressure, Adaptation, Biotechnological potential

43 **Introduction**

44 Oceans harbor a broad diversity of microorganisms involved in all biogeochemical cycles,
45 from coastal waters to the deep biosphere. If prokaryotic communities have been widely
46 studied over the past several years, there has recently been an increasing interest in the
47 diversity of microbial eukaryotes in extreme environments. Sediments from centimeters to
48 hundreds of meters below the seafloor (1-9) or hydrothermal vents (10-13), have been
49 investigated using both molecular and culture-dependent methods. Such integrated
50 approaches revealed the occurrence and activity of complex fungal communities in marine
51 extreme environments. DNA and RNA fungal signatures but also isolated strains provide
52 provide direct evidence that fungi persist in marine sediments, albeit their role in such
53 ecosystems has not been clearly highlighted. Most of the fungal reads and isolates harvested
54 so far belong to the phylum *Ascomycota* and few representatives belong to the *Basidiomycota*
55 and *Chytridiomycota* phyla (14). If some fungal sequences retrieved represent potentially
56 novel organisms, many of them have close neighbors from terrestrial, fresh and salt water
57 environments (4).

58 So far, within subseafloor sediments, fungi have been identified from few centimeters (1) to
59 1740 meters below the sea floor (15) but we are still eager to have better knowledge about
60 their ability to persist as vegetative spores or to play active role(s). Recent metatranscriptomic
61 analyses suggest that fungi, if occurring in lower abundance compared to *Bacteria* and
62 *Archaea*, have nevertheless the ability to degrade a variety of organic substrates in deep
63 subseafloor sediments (9). Such interesting observations could be reinforced with the isolation
64 of fungal representatives from the deep biosphere to search for specific adaptation to *in situ*
65 conditions.

66 Here we present the first culture-based analysis of fungal strains isolated from deep
67 subsurface sediments. The purpose of this survey was (i) to determine the distribution pattern
68 of culturable fungal communities along a sediment core, (ii) to characterize their
69 ecophysiological profiles and consequently to assess their ability to support harsh conditions
70 as elevated hydrostatic pressure, high salinity and different ranges of temperatures and (iii) to
71 evaluate their genetic equipment for synthesis of bioactive compounds.

72 As previous studies have revealed the presence of *Bacteria* and *Archaea* in the deep
73 biosphere, many microbial interactions could occur in sediments including those resulting
74 from synthesis of bioactive compounds (from antimicrobials to cell cycle blockers). Marine
75 fungi from coastal waters are well known to synthesize a broad range of bioactive natural
76 compounds with interesting bioactivities (16), with chemolibraries dominated by polyketides,
77 peptides, alkaloids and terpenoids. Consequently, the deep subseafloor may be considered as
78 an untapped reservoir of such microorganisms producing biomolecules that could be useful to
79 develop biotechnological applications.

80 Here we show that some representatives of fungal communities from deep sediments can be
81 isolated using techniques mimicking *in situ* conditions. We report a global description of
82 culturable representatives from deep sediments collected in the Canterbury basin, from an
83 ecological to a biotechnological perspective.

84

85 **Experimental Procedures**

86 **1. Site description and sediment sampling**

87 Sediment samples were obtained from a core collected in the Canterbury basin on the eastern
88 margin of the New Zealand, South Island at site U1352 (44°56'26.62''S; 172°1'36.30''E).
89 This core reaching a depth of 1927.5 meters below the seafloor was drilled during the
90 Integrated Ocean Drilling Program (IODP) Leg 317 expedition with DS *Joides Resolution* at
91 a water depth of 344 meters. Fluorescent microspheres were used as tracers for contamination
92 during drilling. Sampling was processed under strict contamination controls onboard and
93 offshore and only samples with no detectable contamination were used for this study (17).
94 Subsampling was performed onboard under sterile conditions, and only the center parts of
95 unconsolidated sediments and intact pieces of rocks were kept for microbiological analyses,
96 as reported elsewhere (17). Subsamples were immediately transferred into a sterile Schott
97 bottle sealed with a butyl stopper for storage at 4°C under a nitrogen atmosphere for later
98 cultivation. Recent culture-independent study on the same core has revealed the
99 contamination probability of those sediment samples to be, if not absent, surely insignificant
100 (15). For this study, 11 samples of sediment and sedimentary rock collected at depths from 4
101 to 1884 mbsf were analysed (Table 1).
102

103 **2. Enrichment conditions mimicking *in situ* natural environment**

104 Five different media were used for isolation of fungi under atmospheric pressure, Malt Extract
105 Agar, Potato Dextrose Agar, Corn Meal Agar, Sabouraud Agar and Czapek Dox Agar. Media
106 were diluted at 1/5 to simulate low nutrient conditions of deep sediments as described
107 elsewhere (1). Media were performed with and without 3% (w/v) sea salts and complemented
108 with and without antibiotics (500 mg Chloramphenicol, 200 mg Penicillin). Samples 1H3,
109 2H5, 3H5, 4H1, 5H1, 5H3 and 15H4 were incubated at 5°C, 15°C and 25°C, samples 48X3

110 and 88X1 were incubated at 15°C, 25°C and 30°C and samples 99R1 and 144R3 were
111 incubated at 35°C, 45°C and 55°C.
112 Three different media were used for the isolation of fungal strains under elevated hydrostatic
113 pressure, *i.e.* Sabouraud Broth, Marine Broth and Potato Dextrose Broth diluted at 1/5 and
114 complemented or not with sea salts and antibiotics as previously described. Enrichment
115 cultures were performed in aerobic conditions in sterile syringes and media were saturated
116 with dissolved oxygen. Each syringe contained 5ml of media and 200 µl of sediment sample
117 slurry. The syringes were then transferred into a high-pressure incubation system and incubate
118 at different temperatures. Samples 1H3, 2H5, 3H5, 4H1, 5H1 and 5H3 were incubated at
119 25°C and 4 MPa, samples 15H4, 48X3 and 88X1 were incubated at 30°C and 11 MPa and
120 samples 99R1 and 144R3 were incubated at 45°C and 37 MPa. After 14 days of enrichment
121 under hydrostatic pressure to stimulate the growth of fungi, 100 µl of culture were dispensed
122 on agar plates and incubated at the same temperature until fungal growth. For each condition,
123 pure cultures were obtained by streaking and central picking on their respective enrichment
124 media for fungi showing a distinct morphology.
125 Nutrient plates exposed under laminary flow hood during isolation were processed as control.
126 No fungal colonies were obtained on control plates, indicating the absence of aerial
127 contaminations during isolation.
128
129 **3. DNA extraction**

130 DNA extraction was performed by FastDNA Spin Kit (MP Biomedicals) that is specific for
131 fungi and yeasts following the manufacturer's recommendations. DNA quality and quantity
132 was assessed using a NanoDrop Spectrophotometer (NanoDrop Technologies).
133

134 **4. Mini/Micro-Satellite Primed PCR**

135 Primer used for the MSP-PCR was the core sequence of the phage M13
136 (GAGGGTGGCGGTTCT) (18). The PCR was performed in 25µL containing 1X PCR buffer,
137 2mM $MgCl_2$, 0.4mM of each dNTP, 0.8mM of the primer, 1U of Taq Polymerase and 15ng of
138 genomic DNA. Amplification consisting of an initial denaturation step at 95°C for 5min, then
139 by 40 cycles of 45s at 93°C, 60s at 50°C and 60s at 72°C, and a final extension step of 6 min
140 at 72°C (19). A negative control where DNA was replaced by sterile distilled water was also
141 included. Amplified DNA fragments were separated by electrophoresis in 1.4% (w/v) agarose
142 gel (Promega), in 1X Tris–borate–EDTA (TBE) buffer at 85 V for 3.5 h. On each gel, a
143 molecular size marker of 1kb was used for reference (Promega). DNA banding patterns were
144 visualized under UV transillumination and picture files were generated using Quantum ST4
145 (Vilbert Lourmat). Genetic profiles were analyzed using BioNumerics software (version 6.6,
146 Applied Maths). All fingerprints obtained were grouped by similarity using the Dice
147 coefficient and clustering was based on UPGMA methods. The cophenetic correlation
148 coefficient was calculated. Clusters were done using an arbitrary limit at 75% of similarity
149 with minor adjustments ($^{+}/_{-}$ 5%). Some representative strains were selected in each cluster for
150 further sequencing.

151

152 **5. PCR amplification of 18S, ITS and partial 28S/26S of rRNA**

153 18S rRNA gene sequences were amplified with NS1 (5′-GTAGTCATATGCTTGTCTC-3′)
154 and SR6 (5′-AAGTAGAAGTCGTAACAAGG-3′) primers (20) for genetic identification of
155 fungi. Internal Transcribed Spacer and rDNA 28S (partial) were amplified using ITS5 (5′-
156 GGAAGTAAAAGTCGTAACAAGG-3′) and LR6 (5′-CGCCAGTTCTGCTTACC-3′)
157 primers (respectively 20, 21). Amplifications of the D1/D2 region of 26S rDNA were carried

158 out with primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAG-3'), NL4 (5'-
159 GGTCCGTGTTTCAAGACGG-3') and LR6 (5'-CGCCAGTTCTGCTTA CC-3'), NL1 (5'-
160 GCATATCAATAAGCGGAGGAAAAG-3') as described by Gadanho and Sampaio (10). All
161 PCR reactions were performed in 25 µl reaction volumes containing 1X GoTaq Buffer, 0.4
162 mM dNTPs (Promega), 0.36 µM of each primer (Sigma), 2mM of $MgCl_2$, 1 U of GoTaq
163 polymerase (Promega) and 1 µl of genomic DNA. The PCR assay for 18S and ITS started by
164 initial denaturation step at 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C
165 and 2 min at 72°C and a final extension step of 2 min at 72°C before a conservation at 4°C.
166 The PCR assay for 26S started by initial denaturation step at 94°C for 2 min, followed by 30
167 cycles of 15 s at 94°C, 30 s at 54°C and 1 min at 72°C and a final extension step of 2 min at
168 72°C before a conservation at 4°C. A negative control in which DNA was replaced by sterile
169 water was included. PCR products were controlled by electrophoresis in 0.8% (w/v) agarose
170 gel (Promega) in 1X Tris-Borate EDTA (TBE) buffer at 90 V for 90 min and stained with
171 GelRed. DNA banding patterns were visualized under UV transillumination and picture files
172 were generated using Quantum ST4 (Vilbert Lourmat).
173

174 **6. Sequencing and phylogenetic analyses**

175 18S amplicons were sequenced with NS1, NS3 (5′-GCAAGTCTGGTGCCAGCAGCC-3′),
176 NS6 (5′-AACTTAAAGGAATTGACGGAA-3′) (20) and SR6 primers for SSU rDNA gene
177 sequences. For ITS and LSU rDNA, ITS1, ITS4 and LR6 primers were used. The D1/D2
178 region of the 26S rDNA was sequenced using NL1 on the ITS5-NL4 fragments and NL4 on
179 the NL1-LR6 fragments. Sequences were obtained by 'Big Dye Terminator' technology
180 (Applied Biosystems) at the Biogenouest sequencing platform in the "Station Biologique de
181 Roscoff" (http://www.sb-roscoff.fr/SG/). Chromatograms obtained were translated in

182 nucleotidic sequences with BioNumerics software (version 6.6, Applied Maths). After
183 cleaning, sequences were imported to MEGA 5.0 software (22). Each sequence was analyzed
184 using BLAST-N to find GenBank sequences with close hits (23). Similarities between
185 sequences were assessed using pairwise distance calculation with MEGA 5.0. The sequences
186 were trimmed to ensure that all sequences had the same start and end-point. All the sequences
187 were aligned using ClustalW (version 1.83) (24). Phylogenetic tree topology was realized
188 using Maximum likelihood method with 500 bootstrap iterations, through MEGA 5.0
189 software (22).
190

191 **7. Ecophysiological characterization**

192 This method was performed according to Joubert *et al.* (25). Briefly, suspensions of conidia
193 from selected strains and dilutions were processed in Potato Dextrose Broth (PDB) to reach a
194 final suspension of spores per ml between 1.10^5 and 5.10^5. Then a 96-well plate was filled
195 with those calibrated suspensions (300µL/well) and incubated in a LASER nephelometer
196 (NEPHELOstar PLUS, BMG Labtech) for at least 33h at 15, 25, 30 and 35°C. All
197 experiments were done in triplicate. Growth rate (kRNU/hour) and lag time (hour) were
198 determined for each culture condition: (i) 0, 1.5, 3, 4.5% of sea salts for each temperatures.
199 Different ratios (µmax 1.5% / µmax 0%, µmax 3% / µmax 0% and µmax 4.5% / µmax 0%)
200 were then determined to highlight the adaptation of fungal strains to subsurface *in situ*
201 conditions (3% salinity all along the core and increasing temperatures, *i.e.* around 30-50°C
202 per kilometer).

203

204 **8. Occurrence of type I and III PKS, NRPS, hybrids PKS/NRPS and TPS genes**

205 PKS I, PKS III, NRPS, hybrids PKS/NRPS and TPS genes were amplified using following
206 degenerated primers pairs with dInosine modifications symbolized as "**i**": (i) KAF1
207 (GARKS**i**CAYGG**i**AC**i**GG**i**AC) and KAR1 (CCAYTG**i**GC**i**CCRTG**i**CC**i**GARAA) (26)
208 primers were used for amplification of type I PKS, (ii) CHS1
209 (GAYTGGGC**i**VTNCAYCCBGG**i**GGD) and CHS2
210 (YTCNAYNKTRAKVCC**i**GGVCCRAA) primers for type III PKS, (iii) XKS1
211 (TTYGAYGC**i**BC**i**TTYTTYRA) and XKS2 (CRTT**i**GY**i**CC**i**CYDAA**i**CCAAA) (26) primers
212 for hybrids, (iv) AUG003 (CCGGCACCACCGGNAARCCHAA) and AUG007
213 (GCTGCATGGCGGTGATGSWRTSNCCBCC) (27) primers for NRPS and (v) TPS1
214 (GC**i**TAYGAYAC**i**GC**i**TGGGT) and TPS 2 (RAA**i**GCAT**i**GC**i**GTRTCRTC) (28) primers for
215 TPS. Primers targeting type III PKS were specifically designed for this study using ClustalW
216 (version 1.83) (24). We have selected 18 fungal strains on NCBI database known to have
217 those genes. Each degenerated primer was designed against two conserved domains. All PCR
218 reactions were performed in 25µL containing 1X PCR buffer, 2mM $MgCl_2$, 0.4mM of each
219 dNTP (Promega), 1µM of each primer (Eurogentec), 1U of GoTaq Polymerase (Promega) and
220 100ng of genomic DNA. PCR assay started by initial denaturation step at 98°C for 2 min,
221 followed by 5 cycles of 98°C for 1 min, 45°C for 1 min and 72°C for 2 min followed by 30
222 cycles of 98°C for 1 min, 50°C for 1 min and 72°C for 2 min, and a final extension step for 10
223 min at 72°C. NRPS and TPS amplification were processed with slight modifications: (i) 2 min
224 at 98°C, 35 cycles of 1 min at 98°C, 1 min at 50°C and 2 min at 72°C and 10 min at 72°C
225 (NRPS) and (ii) primer concentration of 0.6 µM and different hybridization temperature of
226 48°C in the first 5 cycles and then 51°C for the other 30 cycles (TPS). For each experiment,
227 we included two positive controls and two negative controls. For positive controls, we used
228 the DNA extracted from *Aspergillus flavus* (UBOCC–A-10181) and *Penicillium chrysogenum*

229 (CBS 306.48) known to have all genes in their genomes. For negative controls, we used DNA
230 extracted from *Saccharomyces cerevisiae* (UBOCC–A-201006) as no gene was ever detected
231 in its genome (30). The other negative control was sterile water instead of DNA.

232

233 **Results**

234 **1. Culturable fungi isolated**

235 A total of 11 samples from 4 to 1884 mbsf, *i.e.* meters below the seafloor (Table 1), were
236 processed for fungal growth of which 10 (91%) yielded to isolation of fungi at different rate
237 (Fig. 1). Under expected *in situ* hydrostatic pressure, 24 syringes were processed per sample
238 including different conditions of temperature (1 per sample according to the expected *in situ*
239 temperature), salinity (0% and 3%), nutrient (3 media), with and without antibiotics and
240 duplicate. Under atmospheric pressure, 120 Petri dishes were processed per sample including
241 different conditions of temperature (3 per sample according to the expected *in situ*
242 temperature), salinity (0% and 3%), nutrient (5 media), with and without antibiotics and
243 duplicate. The final number of fungi obtained was 183 and their distribution across the
244 sediment core is shown in Table 1. Fungi were not cultivated at the same rate at all depths
245 studied. The greatest numbers were found in sediments at 4 and 12 mbsf and from 25 to 37
246 mbsf. Only few species were isolated from the deepest layers at 1478 to 1884 mbsf in
247 sedimentary rock (Fig. 1). Results were analyzed using two-way analysis of variance
248 (ANOVA) to assess the strong significant effect of depth on isolation rate ($F = 3.48$, p-value <
249 0.001). The Shannon index, which is a well-known parameter to express the diversity of
250 communities, was calculated for each depth and corroborates these findings with 34 mbsf as
251 the most complex depth sample. Thus, sediment depth seemed to have a clear influence on

252 isolation and confirms that fungi were mostly present at specific depth along the core and
253 even absent at deepest layer. Indeed, only one strain was isolated from 1884 mbsf and no
254 fungus was ever isolated at 1478 mbsf. This dependence strongly statistically supports the
255 idea that our isolates were not contaminants.

256 Morphological characteristics allowed to separate filamentous fungal forms from unicellular
257 yeasts. Observations revealed branching hyphae and spores for filamentous fungi (124
258 isolates) and budding cells for yeasts (59 isolates). Considering salinity, 86 strains were
259 isolated on culture media complemented with 3% sea salts and 97 without sea salts.
260 Regarding the effect of hydrostatic pressure, 37 strains were isolated under atmospheric
261 pressure and 146 after enrichment under hydrostatic pressure. Among the different conditions
262 used to isolate deep-sea fungi, hydrostatic pressure appeared to be an important parameter to
263 retrieve a significant number of fungal strains. All fungal strains have been deposited in the
264 UBO Culture Collection with a specific UBOCC number (Table S1).

265

266 **2. Dereplication and phylogenetic diversity**

267 To assess the diversity of the isolates, MSP-PCR fingerprints were obtained for all strains. We
268 used similarity clusters to generate two dendrograms, one for filamentous fungi and one for
269 yeasts. Using such strategy, a total of 57 clusters was established, 48 for filamentous fungi
270 and 9 for yeasts as shown in figure S1 and S2. The minor fingerprint variability found within
271 MSP-PCR clusters was defined as intraspecific heterogeneity. Several representatives from
272 each cluster were selected for identification using SSU, ITS and partial LSU rRNA
273 sequencing, representing 72 filamentous and 40 yeast strains sequenced, representing about
274 60% of the whole culture collection.

275 *Filamentous fungi*. Based on the gene encoding SSU ribosomal RNA, representatives from
276 each cluster were assigned using BLAST-N (23) and phylogenetic analysis was processed.
277 When sequences from our culture collection could not be identified using 18S rRNA genes,
278 complementary genetic analyses were performed using ITS and partial 28S rRNA genes.
279 Filamentous fungal strains were distributed among *Ascomycota* and *Basidiomycota* with a
280 dominance of *Ascomycota* representing more than 89% of our culture collection. Based on
281 18S, ITS and 28S sequence analysis, isolates belonging to *Ascomycota* were clustered into 15
282 genera: *Acremonium, Aspergillus, Cladophialophora, Cladosporium, Cordyceps, Eurotium,*
283 *Exophiala, Fusarium, Microascus, Oidiodendron, Paecilomyces, Penicillium, Phialophora,*
284 *Purpureocillium, Sarocladium* and isolates belonging to *Basidiomycota* were clustered into 3
285 genera: *Bjerkandera, Sistotrema, Trametes* and 1 uncultured *Agaricomycetes* (Fig. 2.).

286 *Yeasts*. Sequence analysis of the D1/D2 domain of the 26S rRNA gene allowed to process
287 phylogenetic analysis and revealed yeasts distributed among ascomycetous *Meyerozyma* and
288 basidiomycetous *Rhodotorula* and *Bullera*. With 45 isolates, the *Basidiomycota* phylum
289 represents more than 76% of our yeast culture collection (Fig. 3.).

290

291 **3. Ecophysiological characterization**

292 Laser nephelometry was used as an high-throughput screening method for the
293 ecophysiological characterization of the fungal strains isolated from deep subsurface
294 sediments. Different filamentous fungal strains from the same cluster, but obtained at
295 different depth layers, were selected: 5 *Fusarium* sp. from 4 to 765 mbsf, 4 *Penicillium* sp.
296 from 12 to 403 mbsf, 3 *Paecilomyces* from 4 to 34 mbsf, 2 *Exophiala* sp. from 12 to 34 mbsf
297 and 1 *Acremonium* sp. at 25 mbsf. Maximum specific growth rate (μmax) and lag times were

298 calculated for all conditions (4 temperatures and 4 salinities) and different ratios were finally
299 determined to get ecophysiological information out.

300 Based on the data obtained (Fig. 4.), most of the strains were defined as non-halophiles
301 (maximal growth without sea salts and a decreasing growth rate with increasing
302 concentrations of sea salts in media) or halotolerant (able to grow in the absence as well as in
303 the presence of sea salts) at 25°C and 30°C. Unusable information was obtained at 15°C as
304 most of the strains were still on lag phase after 35h in the nephelometric reader. Only one
305 *Fusarium* sp. collected at 4 mbsf was able to grow at 15°C in less than 35h and displays the
306 same general trend as the 25°C one.

307 Strains affiliated to *Fusarium* sp. were defined as non-halophiles at 25 and 30°C with the
308 same general trends for all sampling depths. Strains affiliated to *Penicillium* sp. were defined
309 as halotolerant at 25°C with a better growth at 3% sea salts. Strains affiliated to *Paecilomyces*
310 sp., *Exophiala* sp. and *Acremonium* sp. display the same pattern at 25°C and are all defined as
311 non-halophiles. Interestingly, at 30°C, these strains could be defined as halotolerant and even
312 halophiles, respectively for the deepest *Exophiala* sp. and the *Acremonium* strains. This
313 ecophysiological shift is clearly emphasized at 35°C with all selected strains that were defined
314 as halophiles at 35°C or even halotolerant for the 25 mbsf *Paecilomyces* sp. Interestingly, this
315 shift was much more significant with the deepest *Penicillium* sp. (137 and 403 mbsf). Such a
316 different ecophysiological behavior for deepest filamentous fungal strains was confirmed with
317 the determination of lag times. Indeed, lag times trends were similar at different salinities for
318 all strains except the deepest *Fusarium* sp. (765 mbsf) and the deepest *Penicillium* sp. (137
319 and 403 mbsf) with lower lag time values at increasing salinities (Fig S3).

320 Different yeasts from the same cluster but from different sampling depths were also selected
321 for ecophysiological analysis. Using the same methodology, maximum specific growth rates
322 were calculated for all conditions (1 temperature and 4 salinities). All strains affiliated to
323 *Rhodotorula* sp. were defined as non-halophiles. Indeed, growth rates decreased when sea
324 salts concentration increased. Strains affiliated to *Meyerozyma* sp. were much more
325 diversified, based on their behaviour. Some strains were non-halophiles (3.76 and 24.6 mbsf),
326 some were halotolerant (37.1 and 137.13 mbsf) and one was halophile (no growth without sea
327 salts) (21.10 mbsf).

328 The screening strategy used allowed to assess eecophysiological differences between isolated
329 strains using temperature and salinity as important *in situ* parameters. Our results suggest
330 adaptation of fungal strains, possibly from terrestrial origin, to deep subsurface conditions.

331

332 **4. Presence of genes involved in the synthesis of bioactive compounds**

333 The easy access to DNA represents a novel opportunity to incorporate sequence analysis in
334 the discovery process. As nearly 10% of fungal genomes are devoted to secondary
335 metabolism (29), PCR-targeting functional genes to find natural product biosynthetic
336 pathways can be seen as a new way for natural product discovery. This sequence-based
337 approach represents a rational and pragmatic approach to select strains with a clear
338 biotechnological potential using bioinformatics to select possible promising strains.

339 Genes coding type I and III PKS, NRPS, hybrids PKS/NRPS and TPS were investigated in all
340 fungal strain isolated (Fig. 5 and 6) using optimized PCR conditions (DNA concentration,
341 annealing temperature, time of elongation,…). Among the 124 filamentous fungi isolated, all
342 harbored at least one of those genes: 6 strains (4.8%) had 1 gene, 23 (18.5%) had 2 genes, 30

343 (24.2%) had 3 genes, 53 (42.7%) had 4 genes and 12 (20.3%) had all targeted genes (Fig. 5).
344 Among the 59 yeasts isolated, 52 harbored at least one of those genes: 16 strains (27.1%) had
345 1 gene, 7 (11.8%) had 2 genes, 12 (20.3%) had 3 genes, 17 (28.8%) had 4 genes. Seven yeasts
346 without any targeted genes have been retrieved (Fig. 6). Contrary to filamentous fungi with 90
347 strains on 124, yeasts did not harbor any positive strain for hybrids PKS/NRPS. Such results
348 appear consistent with previous work indicating the absence of hybrids PKS/NRPS genes in
349 yeast genomes (30) Using such strategy, filamentous fungal strains from deep sediments
350 appeared to have a greater potential for synthesis of bioactive compounds compared to yeasts.

351 Relative occurrence of PKSI, PKSIII, NRPS, Hybrids and TPS genes depending on depth was
352 analyzed (Table S2), from 4 to 765 mbsf (the unique strain isolated from 1884 mbsf was not
353 integrated to this analysis). If no trend was ever visualized for yeasts, filamentous fungal
354 strains from deeper layers seemed to harbor few genes compared to strains from shallow
355 depths. Indeed, some genetic differences were assessed for several strains isolated from
356 different depths. For example, strains affiliated to *Penicillium*, *Fusarium*, *Paecilomyces* and
357 *Rhodotorula* from shallow depths all harbor type III PKS in their genomes contrary to deep
358 layers ones (Fig. 5 and 6).

359

360 **Discussion**

361 The aim of this study was to assess the presence of culturable fungal communities occurring
362 in deep marine subsurface sediments. We studied subsurface sediments collected in the
363 Canterbury basin at 11 different depths from 4 mbsf to 1884 mbsf. The resulting culture
364 collection of filamentous fungi and yeasts provides a broader view of the culturable part of

365 fungal communities colonizing the subseafloor and describes their specificities as taxonomic
366 diversity, ecophysiology and genetic equipment to synthetize bioactive compounds.

367

368 **1. Controlling contamination**

369 Potential microbiological contamination of samples was analyzed during IODP 317
370 Expedition. Standardized sampling procedures and controls have been previously described
371 (15). All manipulations have been carried out in a laminary flow-hood with sterile equipment
372 to avoid contamination. Considering our results, we can observe that 1 (9%) sample processed
373 was sterile for fungi (Fig. 1). No fungi were ever isolated from one depth (1478 mbsf)
374 although the same methods were employed for sampling and isolation. Our ANOVA analysis
375 and Shannon diversity indices have clearly indicated that our isolates were dependent on
376 sampling depths. All these results strongly support the idea that those fungi were not
377 anthropogenic contaminants. The high recovery of fungal strains using elevated hydrostatic
378 pressure enrichments indicated that fungi retrieved were able to grow under pressure and tend
379 to point out that the establish culture collection established consists of indigenous microbial
380 communities. All our results suggest that sampling methods and materials used were not a
381 root of anthropogenic contaminations.

382

383 **2. Distribution pattern of fungal communities**

384 If fungal strains exhibited a quite low overall phylogenetic diversity, distribution of fungal
385 species was greater in the first layers up to 37 mbsf with 25 different species representing
386 more than 92% of the diversity. In deeper samples, only 8 different species were observed

387 which represent about 30% of the diversity including 3 taxa identified only at more than 37
388 mbsf (Fig. 1). This distribution pattern could be explained by the reduction of organic matter
389 accessibility. Indeed, the organic matter quality changed from relatively labile material in
390 shallow sediments to more stable protokerogen at higher sediment depth (15). Among the 27
391 species observed, a maximum of 7 species are shared between 2 depths. Indeed, the
392 filamentous fungi *Acremonium* sp., *Cladophialophora bantiana*, *Penicillium chrysogenum*,
393 *Penicillium* sp., and the yeasts *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa*,
394 are retrieved in 34 mbsf and 37 mbsf samples. Interestingly, *Phialophora* sp.,
395 *Purpureocillium lilacinum, Trametes versicolor* and some *Agaricomycetes* sp. were only
396 isolated at 34 mbsf and seem to be depth-dependent. Beyond 765 mbsf, diversity decreased
397 considerably with only 4 different species observed, including strains belonging to the genus
398 *Fusarium*, one strain identified as *Oidiodendron griseum*, only isolated at 765 mbsf, the red
399 yeast *Rhodotorula mucilaginosa* and one strain isolated at 1884 mbsf, *Sistotrema*
400 *brinkmannii*, also retrieved between 34 and 403 mbsf.

401

402 **3. Diversity of fungal communities**

403 *Ascomycota* dominated among the filamentous fungi whereas *Basidiomycota* were dominant
404 among yeasts in the established culture collection. This result is consistent with another
405 phylogenetic analysis of culturable fungi in deep-sea sediments of the Central Indian Basin
406 (31). Among the yeasts, *Rhodotorula mucilaginosa* is the most abundant species isolated from
407 4 to 765 mbsf sample. Widely distributed and common in deep-sea environments, the red
408 yeast *Rhodotorula mucilaginosa* has already been isolated from deep-sea sediments (32, 31).
409 *Bullera unica,* isolated at 12 mbsf, is the only isolate belonging to *Tremellomycetes.*
410 Interestingly, in a previous study, *Bullera unica* was isolated from New-Zealand seawater

411 (33). *Meyerozyma guilliermondii* was the only ascomycetous yeasts in our culture collection,
412 isolated from 4 to 137 mbsf sample. Culturable representatives of this opportunistic pathogen
413 have been detected in marine environments as hydrothermal vents (11) or deep-sea volcano
414 (34). The yeasts, previously suggested as dominant fungal forms in fungal-specific clone
415 library from water column and marine sediment environments (35), seem to be able to adapt
416 and colonize the deep subseafloor. Our results suggest that filamentous fungi are more diverse
417 in marine habitats than previously suggested (35) with lineages within the *Pezizomycotina* and
418 *Agaricomycotina.* In coherence with molecular studies, *Eurotiomycetes* and *Sordariomycetes*
419 are the most frequently detected fungal taxa from deep-sea environments (14). Among the
420 *Sordariomycetes*, *Fusarium oxysporum* is the most abundant taxa. *F. oxysporum* has already
421 demonstrated its ability to grow in marine conditions (36) and was known as a denitrifying
422 fungus in marine sediments (37). *F. solani* was isolated from a sample collected at 400 mbsf.
423 This result suggests that *F. solani* may survive on buried organic matter at great sediment
424 depths. Some representatives of *Acremonium* sp. have been isolated between 21 and 37 mbsf
425 and have already been reported in several marine environments, including deep-sea sediments
426 (31, 6, 36). *Sarocladium* sp. isolated between 4 and 37 mbsf was also isolated from brine
427 sediments (38) and could be adapted to the salty environments. Strains affiliated to the genus
428 *Paecilomyces* were isolated at 4, 25 and 34 mbsf and previously isolated from deep-sea coral
429 (39) and from the first cm of coastal marine sediments off Goa (37). *Microascus cirrosus,*
430 isolated at 12 mbsf, is known as pathogen in terrestrial environment. In marine environment,
431 species of the genus *Microascus* may be opportunistic pathogens of deep-sea animals, as
432 corals (40). Less represented*, Phialophora* sp., *Purpureocillium lilacinum, Cordyceps*
433 *confragosa* and *Oidiodendron griseum,* were isolated only once at 34, 34, 37 and 765 mbsf,
434 respectively. Among the *Eurotiomycetes,* the genus *Penicillium* was the most frequently

435 isolated, between 4 and 403 mbsf. 14 fungal strains of *Penicillium chrysogenum* have been
436 isolated and have been described as the dominant phylotypes among the *Ascomycota* in DNA-
437 based clones library, in deep-sea methane cold-seep sediments (5). One of the most common
438 terrestrial fungal genus *Aspergillus* has been recovered by culture-dependent methods from
439 marine habitats (11, 1). *Aspergillus* was also present in our culture collection among
440 *Aspergillus fumigatus,* isolated from 137 and 403 mbsf samples and *Aspergillus terreus,*
441 isolated in the first layer at 4 mbsf. *Aspergillus terreus* from the Central Indian Basin was
442 shown being able to germination under hydrostatic pressure and authors suggest that such
443 terrestrial species transported to the deep-sea were initially stressed by deep-sea conditions
444 but may gradually adapt themselves for growth under these extreme conditions (1). *Eurotium*
445 *herbariorum* is the least abundant taxon from the class of *Eurotiomycetes* with 2 strains
446 isolated at 37 mbsf. *E. herbariorum* has been isolated in other extreme environments as
447 hypersaline waters of solar salterns (41), hypersaline Dead Sea (42), hydrothermal sediments
448 (11) or deep-sea sediments (7). In salterns environments, *E. herbariorum* was characterized as
449 a putative member of the indigenous fungal community. Class of *Dothideomycetes* was
450 represented by 3 isolates belonging to the genus *Cladosporium*, isolated in 34 and 37 mbsf
451 samples. Among the *Basidiomycota, Agaricomycetes* is the most dominant with strains close
452 to *Bjerkandera* sp., *Sistotrema brinkmannii*, *Trametes versicolor*, and some strains close to
453 uncultured *Agaricomycetes*. *Bjerkandera* sp. was isolated at 4 mbsf, *Trametes versicolor* at 34
454 mbsf and *Sistotrema brinkmannii* from 34 to the deeper sample at 1884 mbsf. Although the
455 cultivable representatives *Agaricomycetes* are scarce in marine environment (34), molecular
456 signatures of *Agaricomycetes* have also been detected in deep-sea environments (12, 5, 7, 43)
457 and have been identified as the dominant fungal class in mangrove (44). All of the fungal taxa
458 isolated from Canterbury basin are well known in terrestrial environment, raising ecological

459 questions regarding the abilities of terrestrial fungi to adapt to deep subsurface conditions as
460 confinement, pressure, temperature, refractory organic matter...

461

462 **4. Adaptation**

463 Limits to habitability in the deep subseafloor sediments are set by a variety of physical and
464 chemical properties acting both individually and in combination. Low nutrient availability,
465 salinity, hydrostatic and lithostatic pressures, pH, porosity, temperature, energy… could be
466 viewed as a set of extreme parameters influencing life. Using microbial ecology criteria, the
467 deep subsurface is defined as the sediment layers with distinct microbial communities that
468 lack a microbial imprint of water column communities (45). As many terrestrial
469 representatives, in particular fungi, have been detected in deep sediments, the idea is now to
470 answer whether terrestrial microorganisms occurring in this ecosystem are specifically
471 adapted to extreme *in situ* conditions. In our study, we decided (i) to use elevated hydrostatic
472 pressure, as the most significant physical parameter in the abyss (46), to enhance culture
473 efficiency and (ii) to process ecophysiological analyses using temperature and salinity as
474 significant parameters in the deep subseafloor.

475 Hydrostatic pressure represents an important parameter for fungal growth and appeared as an
476 efficient parameter to enhance number of fungal strains in culture collection. Around 80% of
477 our strains were isolated with a preliminary enrichment under elevated hydrostatic pressure.
478 Such enrichment step appears as a growth enhancer and indicates that elevated hydrostatic
479 and lithostatic pressures in deep sediments do not curb the germination and growth of fungi in
480 the deep subseafloor. Previous works have shown the culture efficiency of filamentous fungi
481 and yeasts under elevated hydrostatic pressure (47, 48, 1, 31, 7). Although, no true piezophilic

482 fungi have been reported to date, some fungi are able to develop some mechanisms of
483 adaptation. For example, the well-known yeast *Saccharomyces cerevisiae* is able to modify its
484 membrane composition in order to tolerate high hydrostatic pressure, by increasing the
485 proportion of unsaturated fatty acids and ergosterol (49). This could be viewed as a strong
486 capacity of fungi to adapt to different kind of environmental conditions, even true terrestrial
487 strains, as *S. cerevisiae*.

488 Temperature and salinity are two significant parameters which were used here to propose
489 hypothesis on adaptation of fungi to deep sediment *in situ* conditions. Indeed, temperature
490 increases along the sediment core at a rate of 30-50°C per kilometer. Temperature at the
491 bottom of the hole was estimated to be in the range of 60-100°C (15). Salinities in samples
492 near the seafloor are slightly lower than normal seawater at 3.3%, rapidly decline to 3.0% at
493 28 mbsf, and remain relatively constant at 2.9–3.1% in the rest of the section analyzed, to
494 1400 mbsf (17). Ecophysiological analyses processed on fungal strains revealed shifts along
495 the core. Most of the filamentous fungal strains analyzed present a slight trend consisting in a
496 shift from non-halophilic to halotolerant styles when incubation temperature increased from
497 25 to 35°C. Only the deepest *Fusarium* and *Penicillium* present a complete shift to halophily
498 when temperature increased. Such a shift from terrestrially-adapted to marine-adapted styles
499 along the core may indicate a kind of adaptation from shallow layers fungi not specifically
500 adapted but able to survive to deep layers fungi more adapted to deep subseafloor conditions,
501 *i.e.* higher temperatures and higher salinity than terrestrial ecosystems. Ecophysiological
502 studies on deep subseafloor prokaryotes are scarce. Biddle *et al.* (50) have used a low salt
503 medium (R2A) to culture bacterial strains from deep sediments of the Peru Margin and
504 obtained colonies only at shallow depth layers. This may indicate the occurrence of non-
505 marine strains in the first layers and endemic or adapted strains at deeper layers. However, it

506 can also only reflect the decrease in isolation success with depth. However, some generalist
507 bacteria isolated from different subseafloor sediments were adapted to a broader spectrum of
508 environmental conditions compared to their surface counterparts (51). Such results strongly
509 supports our conclusions.

510 Fungi isolated in this study are ubiquitous but some of them seem to be buried but alive and
511 thus may undoubtly have a role in the deep biosphere. Fungi in the deep subseafloor may
512 harbor different lifestyles from dormancy to adaptation and corroborate information available
513 for prokaryotes. Indeed, bacterial endospores are as abundant as vegetative prokaryotic cells
514 in the deep biosphere (52). Recenlty, Jørgensen (53) proposed that most of the deep
515 subseafloor microbial cells were on physiological standby and that only few cells may be
516 active at any given time. More over, the metabolic potential of long-buried microbes could be
517 activated at much higher rates when they emerge into a moderate environment. Such
518 hypothesis fits well with our findings that some fungi may set up a wide range of adaptations
519 to handle different physical and chemical stress and some others might be microbial zombies,
520 as proposed by Colwell and D'Hondt (54). First hints were here obtained but complementary
521 analyses must be processed to better understand the metabolic capabilities of deep subseafloor
522 fungi using genomic, transcriptomic and proteomic-based analyses.

523

524 **5. Biotechnological potential of deep sediment fungal strains**

525 Many of the active compounds used in medicine are based on or related to natural substances
526 obtained from plants, microorganisms or even animals (55), and most of these molecules have
527 been isolated and characterized from terrestrial organisms (56). Today, one major issue is to
528 avoid the re-isolation of well-known metabolites representing a waste of time and money. To

529 enhance chances to discover novel molecules, it is critically important (i) to search for
530 untapped biological resources in untargeted ecological niches and (ii) to rationalize the search
531 for bioactive compounds with pragmatic approaches rather than traditional approaches based
532 on serendipity. The marine environment, which covers 70% of the earth surface and contains
533 the greatest diversity of life on Earth, remains a largely unexplored biome. The oceans are the
534 vastest resource to discover novel chemical structures with novel modes of action. More and
535 more bioactive compounds are searched among marine (micro)organisms (57, 58) with some
536 research dedicated to marine fungi in order to find novel compounds such as antibiotics,
537 anticancer,... (16). As a pragmatic approach, many *in silico* tools are now used to detect genes
538 encoding enzymes involved in the synthesis of such secondary metabolite compounds, *e.g.*
539 NapDos, MAPSI or SearchPKS (59). The idea is to select interesting (micro)organisms, *i.e.*
540 harboring interesting genes involved in secondary metabolites pathways. In this study, we
541 decided to detect the presence of genes encoding type I and III PKS, NRPS, hybrid PKS /
542 NRPS and TPS as genes of interest to rationalize the search of active natural products (60).
543 We noticed that only 7 yeasts have no targeted genes. All other fungi isolated here have at
544 least one gene involved in secondary metabolites biosynthesis pathways. Such results clearly
545 indicate (i) the high genetic diversity of these strains and (ii) a genetic potential for the
546 synthesis of numerous secondary metabolites with potentially interesting bioactivities, as
547 many of these genes might be responsible for the biosynthesis of natural compounds, helping
548 the fungi to compete with other microorgansisms (26).

549 Strains affiliated to *Penicillium*, *Fusarium*, *Paecilomyces* and *Rhodotorula* have shown
550 genetic differences depending on sampling depth. Strains isolated at shallow depths (from 4 to
551 25 mbsf) harbored type III PKS contrary to the same strains isolated from deeper layers (from
552 25 to 700 mbsf). More detailed genomic-based and/or phylogenetic analyses on these specific

553 genes should be done to sort out information about evolutionary origin. However, as recent
554 studies have shown that secondary metabolism gene clusters evolve rapidly through multiple
555 rearrangements, duplication or losses (63), it could be that these subseafloor fungi have lost
556 some genes to adapt to their environment and may confirmed what was previously obtained
557 regarding ecophysiological adaptation.

558 Our results demonstrate the effectiveness of isolating some representatives of fungal
559 communities occurring in the deep subseafloor and give interesting hints about adaptation of
560 such organisms and also their biotechnological potential. If novel methods have recently
561 allowed to study the genetic capacities of uncultured microorganisms using single-cell
562 genomics and metatranscriptomics, some critical physiological features are currently only
563 obtained using culture-based analyses. The present established culture collection offers future
564 biotechnological opportunities that should be amended by further studies specifically
565 dedicated to the expression of such genes of interest.

566

567 **Conflict of Interest**

568 The authors declare no conflict of interest.

569

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759 **Table and Figure legends**

760

761 **Fig. 1. Species richness along the core.** Shannon diversity indices among sediment samples

762 were calculated allowing the identification of the less and more complex depth samples.

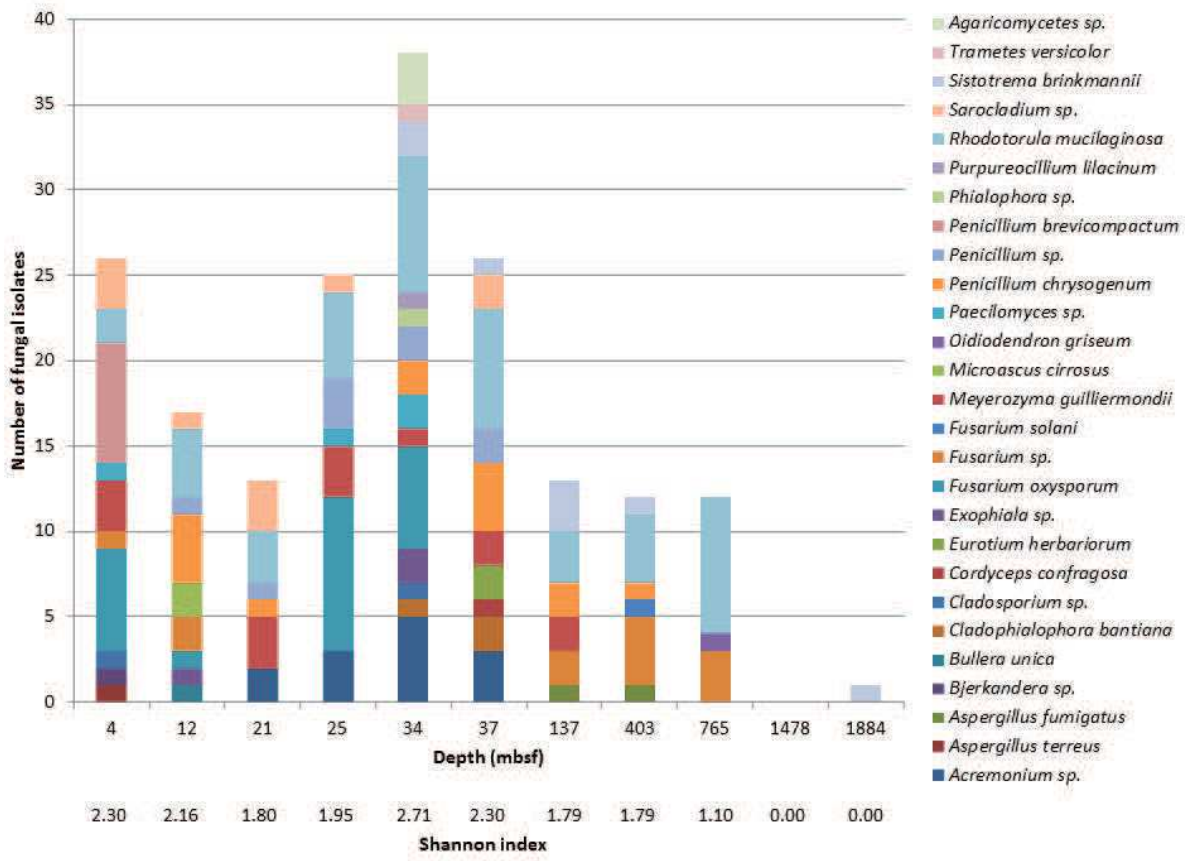

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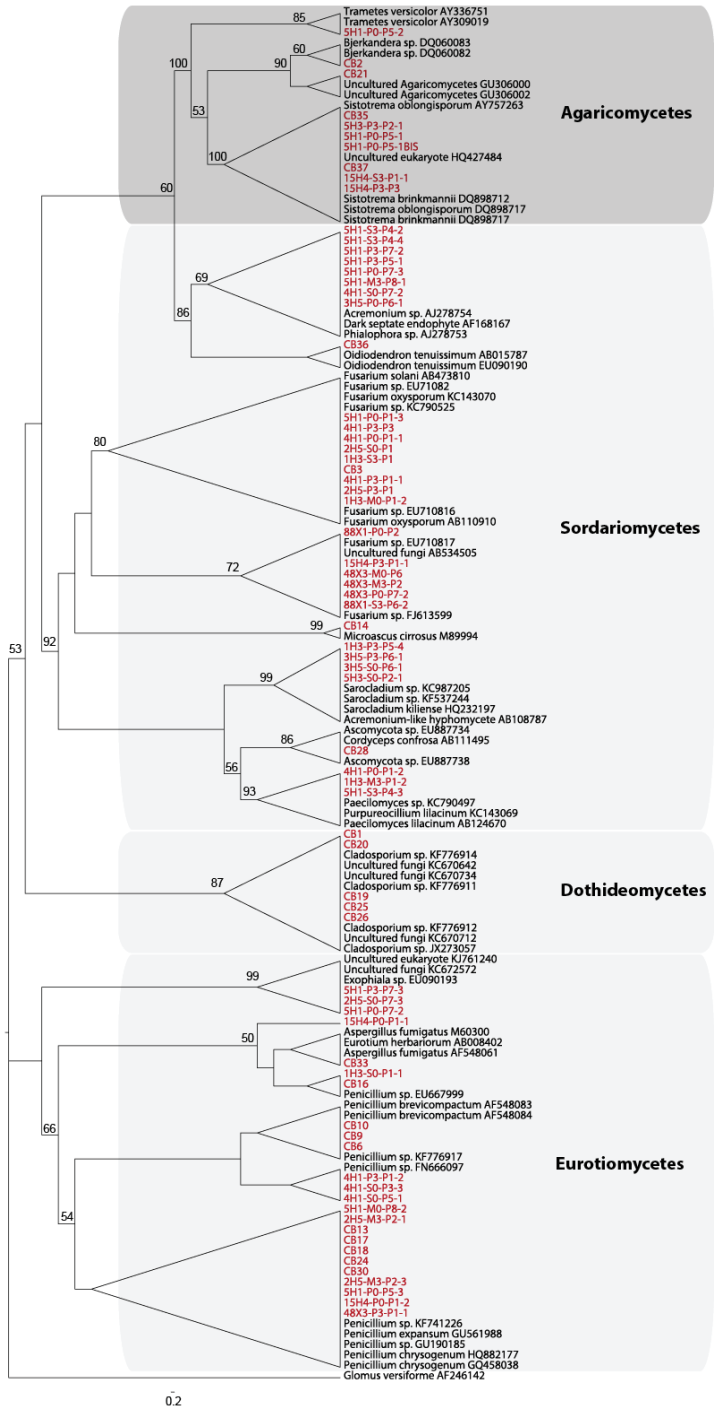
Fig. 2. SSU rRNA phylogenetic positions of deep-sea fungal isolates (coloured terminals). Topology was built using Maximum likelihood method from a ClustalW 1.83 alignment. Bootstrap values greater than 50% are shown at nodes. *Glomus versiforme* (AF246142) belonging to the Glomeromycota phylum was used as outgroup. Dark gray box represents the *Basidiomycota* and gray box the *Ascomycota*.



771 **Fig. 3. Phylogenetic tree of deep-sea yeast isolates (coloured terminals) obtained by**

772 **analysis of the D1/D2 domain of the 26S rRNA gene.** Topology was built using Maximum

773 likekihood method from a ClustalW 1.83 alignment. Bootstrap values greater than 50% are

774 shown at nodes. *Mucor flavus* (EU071390) belonging to the Zygomycota phylum was used as

775 outgroup.

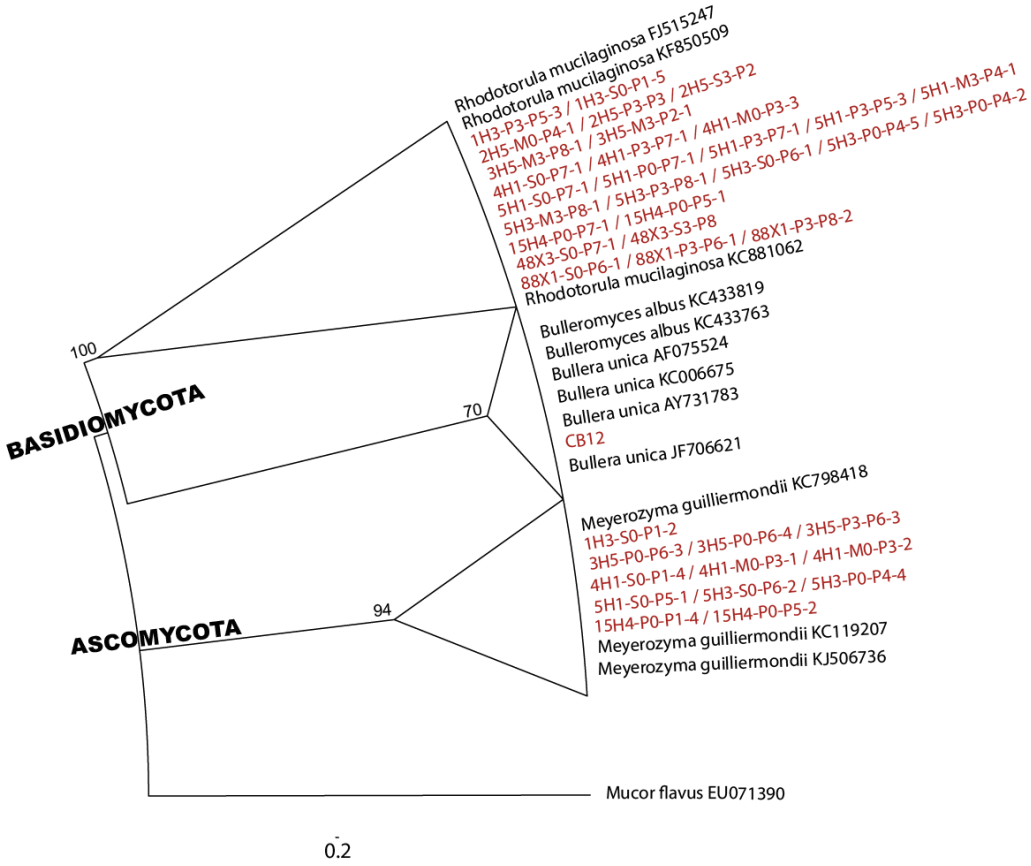


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Fig. 4. Physiological analysis of filamentous fungi (A) and yeast (B) strains. Growth was measured at different temperatures (25°C, 30°C and 35°C), different sea salt concentrations (0, 3 and 4.5%) and general growth trend is shown as 3-part bars representing minimum, medium and maximum growth. (nd: not determined)

(A)

µmax	Temperature	25°C			General trend	30°C			General trend	35°C			General trend
	% Sea salts	1,5 / 0	3 / 0	4,5 / 0		1,5 / 0	3 / 0	4,5 / 0		1,5 / 0	3 / 0	4,5 / 0	
Fusarium sp.	4 mbsf	1,01	0,87	0,71		nd	nd	nd		1,46	1,61	1,29	
	25 mbsf	0,97	0,91	0,82		0,92	nd	nd		nd	nd	nd	
	137 mbsf	1,04	0,81	0,40		0,84	0,71	0,67		1,15	1,27	1,13	
	403 mbsf	1,01	0,85	0,84		0,86	0,82	0,76		nd	nd	nd	
	765 mbsf	0,94	0,81	0,79		nd	nd	nd		1,56	1,71	1,80	
Penicillium sp.	12 mbsf	0,87	0,91	0,74		nd	nd	nd		0,85	1,22	1,55	
	25 mbsf	1,30	1,53	0,94		nd	nd	nd		0,97	1,35	1,19	
	137 mbsf	1,07	0,96	0,91		nd	nd	nd		2,36	4,67	5,71	
	403 mbsf	0,88	0,93	0,81		nd	nd	nd		0,81	1,40	1,72	
Paecilomyces sp.	4 mbsf	0,94	0,84	0,82		nd	nd	nd		3,40	7,46	7,07	
	25 mbsf	0,81	0,73	0,60		1,00	0,96	0,79		1,20	1,17	1,17	
	34 mbsf	0,91	0,84	0,75		0,97	0,96	0,84		nd	nd	nd	
Exophiala sp.	12 mbsf	0,96	0,80	0,65		1,45	0,85	0,72		nd	nd	nd	
	34 mbsf	0,72	0,61	0,56		1,16	1,21	1,05		nd	nd	nd	
Acremonium sp.	25 mbsf	0,88	0,79	0,69		2,79	2,62	3,22		nd	nd	nd	

(B)

□max	Temperature	30°C			General trend
	% Sea salts	1,5 / 0	3 / 0	4,5 / 0	
Rhodotorula mucilaginosa	3.76 mbsf	0,69	0,55	0,42	
	11.60 mbsf	0,65	0,59	0,43	
	21.1 mbsf	0,81	0,58	0,48	
	24.60 mbsf	0,77	0,54	0,45	
	34.10 mbsf	0,77	0,48	0,34	
	37.10 mbsf	1,38	1,37	1,22	
	403.1 mbsf	0,76	0,50	0,40	
	765 mbsf	0,66	0,52	0,38	
Meyerozyma guilliermondii	3.76 mbsf	1,24	1,17	1,10	
	21.10 mbsf	1,08	1,21	1,32	
	24.60 mbsf	1,27	1,19	1,16	
	37.1 mbsf	0,68	0,69	0,37	
	137.13 mbsf	1,39	1,32	1,33	

Fig. 5. Presence/Absence of genes coding type I and III PKS, NRPS, hybrids PKS-NRPS and TPS in filamentous fungi. Data set of filamentous fungal MSP-PCR fingerprints coupled with type I (clear blue), type III (pink) PKS, NRPS (clear green), hybrids PKS/NRPS (dark blue) and TPS (dark green) genes occurrence using aligned multi value bar chart (small bar, only one gene and long bar, several genes). This figure was generated using Interactive Tree of Life v2 (64).

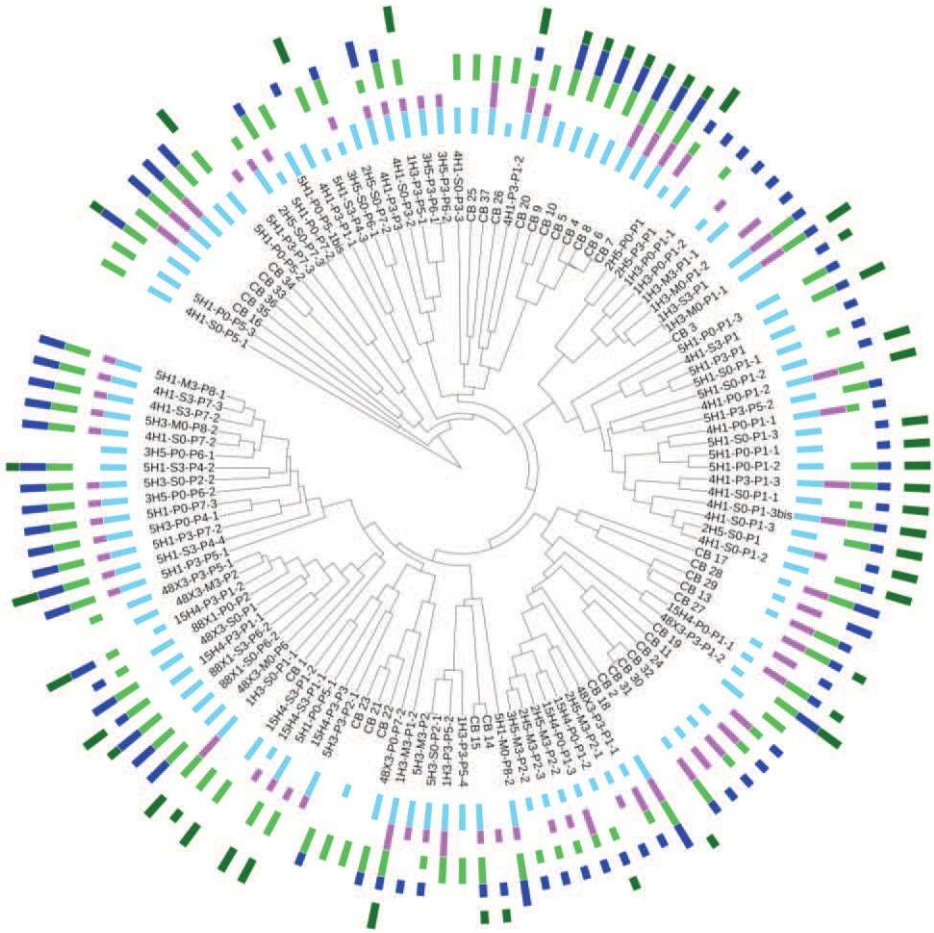
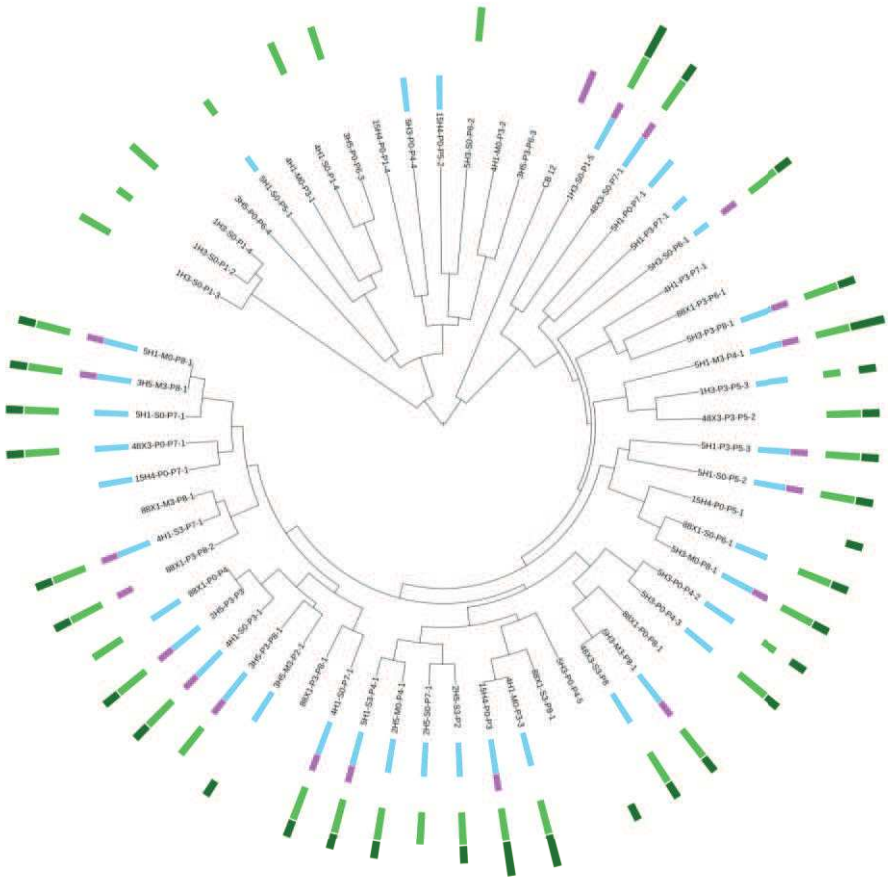


Fig. 6. Presence/Absence of genes coding type I and III PKS, NRPS, hybrids PKS-NRPS and TPS in yeasts. Data set of yeast MSP-PCR fingerprints coupled with type I (clear blue), type III (pink) PKS, NRPS (clear green), hybrids PKS/NRPS (dark blue) and TPS (dark green) genes occurrence using aligned multi value bar chart (small bar, only one gene and long bar, several genes). This figure was generated using Interactive Tree of Life v2 (64).



802 **Table 1. Culture collection of fungi from deep subseafloor sediments.**

Sample ID	Core depth below the seafloor (m)	Total number of genus/strain	Number of genus/strain isolated with enrichment under hydrostatic pressure	Number of genus/strain isolated under atmospheric pressure
1H3	4	9/26	7/16	4/10
2H5	12	7/17	5/12	3/5
3H5	21	5/13	5/12	1/1
4H1	25	7/25	7/25	0
5H1	34	14/38	11/31	4/7
5H3	37	9/26	5/15	4/11
15H4	137	6/13	6/13	0
48X3	403	5/12	4/11	1/1
88X1	765	3/12	2/11	1/1
99R1	1478	0	0	0
144R3	1884	1/1	0	1/1
Total		183	146	37

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Appendixes

Fig. S1. MSP-PCR profiles obtained for filamentous fungi. Representatives of clusters selected for sequencing were identified using the * symbol.

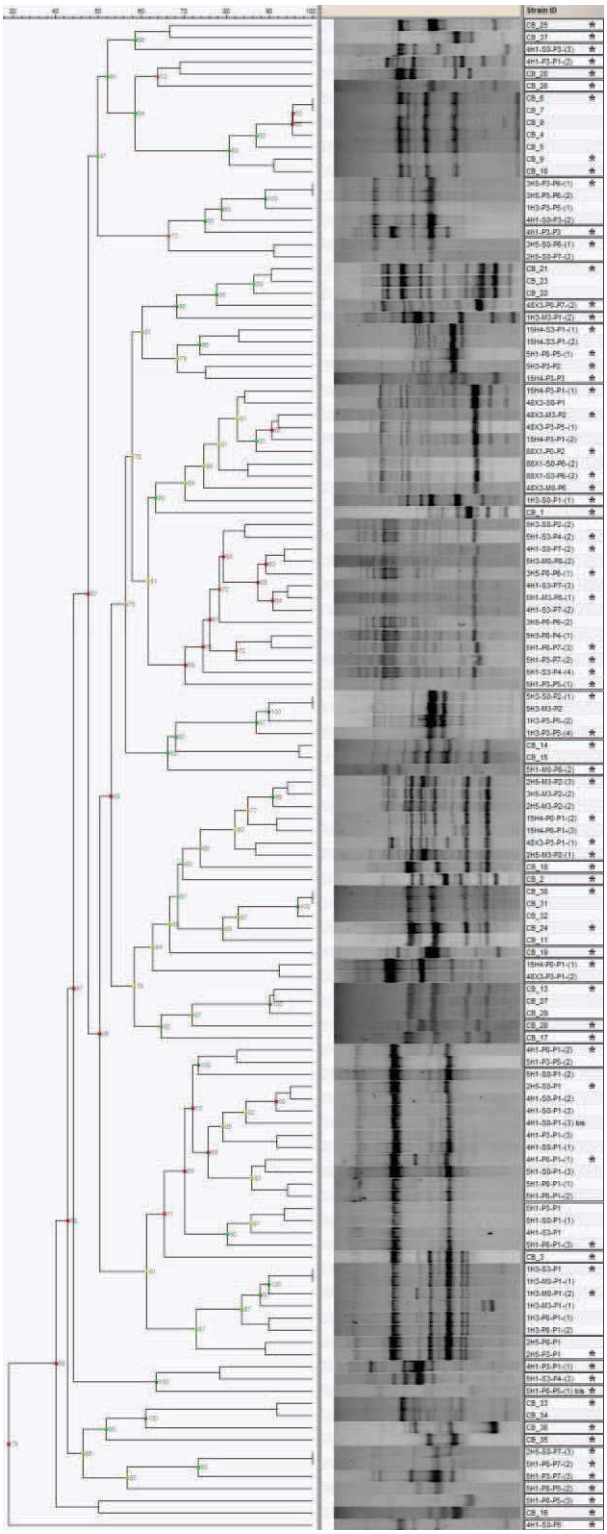

Fig. S2. MSP-PCR profiles obtained for the yeasts. Representatives of clusters selected for sequencing were identified using the * symbol.

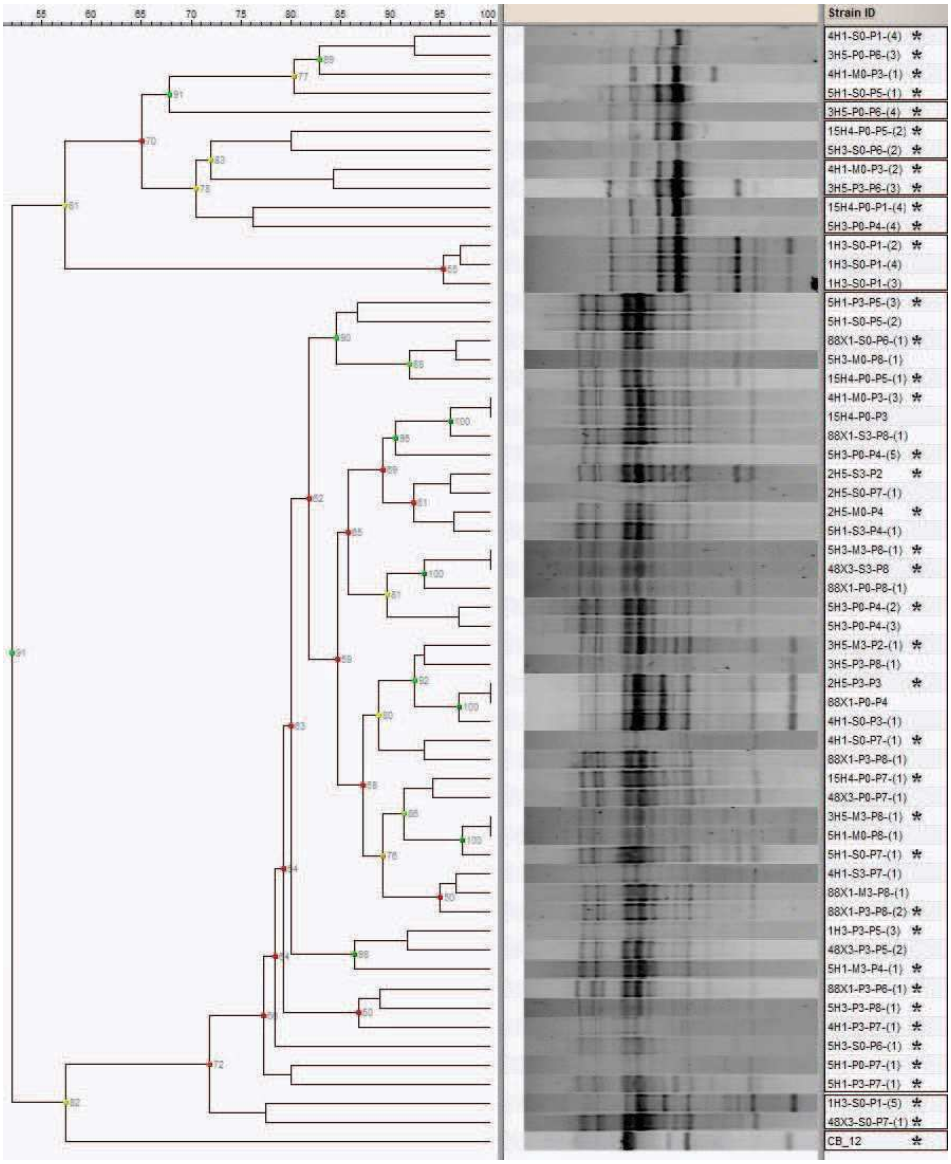

Fig. S3. Lag time for filamentous fungi. (nd: not determined)

lag time	Temperature	25°C			General trend	30°C			General trend	35°C			General trend
	% Sea salts	1,5 / 0	3 / 0	4,5 / 0		1,5 / 0	3 / 0	4,5 / 0		1,5 / 0	3 / 0	4,5 / 0	
Fusarium sp.	4 mbsf	0,99	0,80	0,93						1,00	0,33	0,36	
	25 mbsf	0,92	1,00	1,04		0,87	nd	nd					
	137 mbsf	1,22	1,26	1,29		0,85	0,77	0,92		0,87	0,69	0,69	
	403 mbsf	1,00	1,00	1,16		0,67	0,50	0,50					
	765 mbsf	1,00	0,56	0,70						1,11	1,08	0,91	
Penicillium sp.	12 mbsf	0,78	0,64	0,83						0,92	0,87	0,80	
	25 mbsf	0,92	0,85	1,04						0,96	0,72	0,83	
	137 mbsf	1,07	0,70	0,88						0,95	0,90	0,70	
	403 mbsf	0,76	0,63	0,73						0,94	0,96	1,01	
Paecilomyces sp.	4 mbsf	0,93	0,76	0,93						0,87	0,86	0,77	
	25 mbsf	1,05	1,08	1,10		1,02	0,96	1,20		0,76	0,61	0,61	
	34 mbsf	1,04	1,04	1,10		1,03	0,91	1,27					
Exophiala sp.	12 mbsf	0,96	1,15	1,35		0,81	0,78	1,00					
	34 mbsf	0,96	0,72	1,09		0,89	0,92	1,22					
Acremonium sp.	25 mbsf	0,95	1,07	1,07		0,78	0,70	0,68					

815 **Table S1. Fungal isolates identification.**

UBOCC number	Genus	Species	Sample ID	Accession number		
				18S	ITS	26S
UBOCC-A-114001	*Fusarium*	*oxysporum*	1H3-P0-P1-1			
UBOCC-A-114002	*Fusarium*	*oxysporum*	1H3-P0-P1-2			
UBOCC-A-114003	*Aspergillus*	*terreus*	1H3-S0-P1-1	KM222201	KM232439	
UBOCC-A-214003	*Meyerozyma*	*guilliermondii*	1H3-S0-P1-2	KM222231		KM222311
UBOCC-A-214004	*Meyerozyma*	*guilliermondii*	1H3-S0-P1-3			
UBOCC-A-214005	*Meyerozyma*	*guilliermondii*	1H3-S0-P1-4			
UBOCC-A-214006	*Rhodotorula*	*mucilaginosa*	1H3-S0-P1-5	KM222232		KM222320
UBOCC-A-114004	*Fusarium*	*oxysporum*	1H3-S3-P1	KM222233	KM232440	
UBOCC-A-114005	*Fusarium*	*oxysporum*	1H3-M0-P1-1			
UBOCC-A-114006	*Fusarium*	*oxysporum*	1H3-M0-P1-2	KM222200	KM232436	
UBOCC-A-114007	*Fusarium*	*oxysporum*	1H3-M3-P1-1			
UBOCC-A-114008	*Paecilomyces*	sp.	1H3-M3-P1-2	KM222228	KM232437	
UBOCC-A-114009	*Fusarium*	sp.	2H5-P0-P1			
UBOCC-A-114010	*Fusarium*	sp.	2H5-P3-P1	KM222202	KM232443	
UBOCC-A-114011	*Fusarium*	*oxysporum*	2H5-S0-P1	KM222238	KM232444	
UBOCC-A-114012	*Fusarium*	*oxysporum*	4H1-P0-P1-1	KM222252	KM232449	
UBOCC-A-114013	*Paecilomyces*	sp.	4H1-P0-P1-2	KM222253	KM232450	
UBOCC-A-114014	*Fusarium*	*oxysporum*	4H1-P3-P1-1	KM222203	KM232451	
UBOCC-A-114015	*Penicillium*	sp.	4H1-P3-P1-2	KM222254	KM232452	
UBOCC-A-114016	*Fusarium*	*oxysporum*	4H1-P3-P1-3			
UBOCC-A-114017	*Fusarium*	*oxysporum*	4H1-S0-P1-1			
UBOCC-A-114018	*Fusarium*	*oxysporum*	4H1-S0-P1-2			
UBOCC-A-114019	*Fusarium*	*oxysporum*	4H1-S0-P1-3			
UBOCC-A-114020	*Fusarium*	*oxysporum*	4H1-S0-P1-3BIS			
UBOCC-A-214007	*Meyerozyma*	*guilliermondii*	4H1-S0-P1-4	KM222257		KM222321
UBOCC-A-114021	*Fusarium*	*oxysporum*	4H1-S3-P1			
UBOCC-A-114022	*Fusarium*	*oxysporum*	5H1-P0-P1-1			
UBOCC-A-114023	*Fusarium*	*oxysporum*	5H1-P0-P1-2			

UBOCC-A-114024	*Fusarium*	*oxysporum*	5H1-P0-P1-3	KM222282	KM232459	
UBOCC-A-114025	*Fusarium*	*oxysporum*	5H1-P3-P1			
UBOCC-A-114026	*Fusarium*	*oxysporum*	5H1-S0-P1-1			
UBOCC-A-114027	*Paecilomyces*	sp.	5H1-S0-P1-2			
UBOCC-A-114028	*Fusarium*	*oxysporum*	5H1-S0-P1-3			
UBOCC-A-114029	*Aspergillus*	*fumigatus*	15H4-P0-P1-1	KM222291	KM232473	
UBOCC-A-114030	*Penicillium*	*chrysogenum*	15H4-P0-P1-2	KM222292	KM232474	
UBOCC-A-114031	*Penicillium*	*chrysogenum*	15H4-P0-P1-3			
UBOCC-A-214008	*Meyerozyma*	*guilliermondii*	15H4-P0-P1-4	KM222293		KM222322
UBOCC-A-114032	*Fusarium*	sp.	15H4-P3-P1-1	KM222297	KM232475	
UBOCC-A-114033	*Fusarium*	sp.	15H4-P3-P1-2			
UBOCC-A-114034	*Sistotrema*	*brinkmannii*	15H4-S3-P1-1	KM222299	KM232477	
UBOCC-A-114035	*Sistotrema*	*brinkmannii*	15H4-S3-P1-2			
UBOCC-A-114036	*Penicillium*	*chrysogenum*	48X3-P3-P1-1	KM222303	KM232481	
UBOCC-A-114037	*Aspergillus*	*fumigatus*	48X3-P3-P1-2			
UBOCC-A-114038	*Fusarium*	sp.	48X3-S0-P1			
UBOCC-A-214009	*Rhodotorula*	*mucilaginosa*	2H5-S3-P2	KM222240		KM222323
UBOCC-A-114039	*Penicillium*	*chrysogenum*	2H5-M3-P2-1	KM222235	KM232441	
UBOCC-A-114040	*Penicillium*	*chrysogenum*	2H5-M3-P2-2			
UBOCC-A-114041	*Penicillium*	*chrysogenum*	2H5-M3-P2-3	KM222236	KM232442	
UBOCC-A-214010	*Rhodotorula*	*mucilaginosa*	3H5-M3-P2-1	KM222241		KM222324
UBOCC-A-114042	*Penicillium*	*chrysogenum*	3H5-M3-P2-2			
UBOCC-A-114043	*Sistotrema*	*brinkmannii*	5H3-P3-P2-1	KM222271	KM232472	
UBOCC-A-114044	*Sarocladium*	sp.	5H3-S0-P2-1	KM222288	KM242082	
UBOCC-A-114045	*Acremonium*	sp.	5H3-S0-P2-2			
UBOCC-A-114046	*Sarocladium*	sp.	5H3-M3-P2			
UBOCC-A-114047	*Fusarium*	sp.	48X3-M3-P2	KM222301	KM232479	
UBOCC-A-114048	*Fusarium*	sp.	88X1-P0-P2	KM222306	KM232482	
UBOCC-A-214011	*Rhodotorula*	*mucilaginosa*	2H5-P3-P3	KM222237		KM222325
UBOCC-A-114049	*Fusarium*	*oxysporum*	4H1-P3-P3	KM222255	KM232453	
UBOCC-A-214012	*Rhodotorula*	*mucilaginosa*	4H1-S0-P3-1			
UBOCC-A-114050	*Sarocladium*	sp.	4H1-S0-P3-2			

UBOCC-A-114051	*Penicillium*	sp.	4H1-S0-P3-3	KM222258	KM232454	
UBOCC-A-214013	*Meyerozyma*	*guilliermondii*	4H1-M0-P3-1	KM222249		KM222326
UBOCC-A-214014	*Meyerozyma*	*guilliermondii*	4H1-M0-P3-2	KM222250		KM222327
UBOCC-A-214015	*Rhodotorula*	*mucilaginosa*	4H1-M0-P3-3	KM222251		KM222328
UBOCC-A-214016	*Rhodotorula*	*mucilaginosa*	15H4-P0-P3			
UBOCC-A-114052	*Sistotrema*	*brinkmannii*	15H4-P3-P3	KM222298	KM232476	
UBOCC-A-214017	*Rhodotorula*	*mucilaginosa*	2H5-M0-P4-1	KM222234		KM222329
UBOCC-A-214018	*Rhodotorula*	*mucilaginosa*	5H1-S3-P4-1			
UBOCC-A-114053	*Acremonium*	sp.	5H1-S3-P4-2	KM222278	KM232469	
UBOCC-A-114054	*Purpureocillium*	*lilacinum*	5H1-S3-P4-3	KM222279	KM232470	
UBOCC-A-114055	*Acremonium*	sp.	5H1-S3-P4-4	KM222280	KM232471	
UBOCC-A-214019	*Rhodotorula*	*mucilaginosa*	5H1-M3-P4-1	KM222263		KM222330
UBOCC-A-114056	*Acremonium*	sp.	5H3-P0-P4-1			
UBOCC-A-214020	*Rhodotorula*	*mucilaginosa*	5H3-P0-P4-2	KM222283		KM222331
UBOCC-A-214021	*Rhodotorula*	*mucilaginosa*	5H3-P0-P4-3			
UBOCC-A-214022	*Meyerozyma*	*guilliermondii*	5H3-P0-P4-4	KM222284		KM222332
UBOCC-A-214023	*Rhodotorula*	*mucilaginosa*	5H3-P0-P4-5	KM222285		KM222333
UBOCC-A-214024	*Rhodotorula*	*mucilaginosa*	88X1-P0-P4			
UBOCC-A-114057	*Sarocladium*	sp.	1H3-P3-P5-1			
UBOCC-A-114058	*Sarocladium*	sp.	1H3-P3-P5-2			
UBOCC-A-214025	*Rhodotorula*	*mucilaginosa*	1H3-P3-P5-3	KM222229		KM222334
UBOCC-A-114059	*Sarocladium*	sp.	1H3-P3-P5-4	KM222230	KM232438	
UBOCC-A-114060	*Penicillium*	sp.	4H1-S0-P5-1	KM222259	KM232455	
UBOCC-A-114061	*Sistotrema*	*brinkmannii*	5H1-P0-P5-1	KM232435	KM232460	
UBOCC-A-114062	*Sistotrema*	*brinkmannii*	5H1-P0-P5-1bis	KM222265	KM232461	
UBOCC-A-114063	*Trametes*	*versicolor*	5H1-P0-P5-2	KM222266	KM232462	
UBOCC-A-114064	*Penicillium*	sp.	5H1-P0-P5-3	KM222267	KM232463	
UBOCC-A-114065	*Acremonium*	sp.	5H1-P3-P5-1	KM222272	KM232466	
UBOCC-A-114066	*Paecilomyces*	sp.	5H1-P3-P5-2			
UBOCC-A-214026	*Rhodotorula*	*mucilaginosa*	5H1-P3-P5-3	KM222286		KM222335
UBOCC-A-214027	*Meyerozyma*	*guilliermondii*	5H1-S0-P5-1	KM222276		KM222317
UBOCC-A-214028	*Rhodotorula*	*mucilaginosa*	5H1-S0-P5-2			

UBOCC-A-214029	*Rhodotorula*	*mucilaginosa*	15H4-P0-P5-1	KM222294		KM222315
UBOCC-A-214030	*Meyerozyma*	*guilliermondii*	15H4-P0-P5-2	KM222295		KM222314
UBOCC-A-114067	*Fusarium*	sp.	48X3-P3-P5-1			
UBOCC-A-214031	*Rhodotorula*	*mucilaginosa*	48X3-P3-P5-2			
UBOCC-A-114068	*Acremonium*	sp.	3H5-P0-P6-1	KM222243	KM232446	
UBOCC-A-114069	*Acremonium*	sp.	3H5-P0-P6-2			
UBOCC-A-214032	*Meyerozyma*	*guilliermondii*	3H5-P0-P6-3	KM222244		KM222318
UBOCC-A-214033	*Meyerozyma*	*guilliermondii*	3H5-P0-P6-4	KM222245		KM222319
UBOCC-A-114070	*Sarocladium*	sp.	3H5-P3-P6-1	KM222246	KM232447	
UBOCC-A-114071	*Sarocladium*	sp.	3H5-P3-P6-2			
UBOCC-A-114072	*Meyerozyma*	*guilliermondii*	3H5-P3-P6-3	KM222247		KM222336
UBOCC-A-114073	*Sarocladium*	sp.	3H5-S0-P6-1	KM222248	KM232448	
UBOCC-A-214034	*Rhodotorula*	*mucilaginosa*	5H3-S0-P6-1	KM222289		KM222337
UBOCC-A-214035	*Meyerozyma*	*guilliermondii*	5H3-S0-P6-2	KM222290		KM222316
UBOCC-A-114074	*Fusarium*	sp.	48X3-M0-P6	KM222300	KM232478	
UBOCC-A-214036	*Rhodotorula*	*mucilaginosa*	88X1-P3-P6-1	KM222307		KM222338
UBOCC-A-214037	*Rhodotorula*	*mucilaginosa*	88X1-S0-P6-1	KM222309		KM222339
UBOCC-A-114075	*Fusarium*	sp.	88X1-S0-P6-2			
UBOCC-A-114076	*Fusarium*	sp.	88X1-S3-P6-2	KM222310	KM232483	
UBOCC-A-214038	*Rhodotorula*	*mucilaginosa*	2H5-S0-P7-1			
UBOCC-A-114077	*Sarocladium*	sp.	2H5-S0-P7-2			
UBOCC-A-114078	*Exophiala*	sp.	2H5-S0-P7-3	KM222239	KM232445	
UBOCC-A-214039	*Rhodotorula*	*mucilaginosa*	4H1-P3-P7-1	KM222256		KM222340
UBOCC-A-214040	*Rhodotorula*	*mucilaginosa*	4H1-S0-P7-1	KM222260		KM222341
UBOCC-A-114079	*Acremonium*	sp.	4H1-S0-P7-2	KM222261	KM232456	
UBOCC-A-214041	*Rhodotorula*	*mucilaginosa*	4H1-S3-P7-1			
UBOCC-A-114080	*Acremonium*	sp.	4H1-S3-P7-2			
UBOCC-A-114081	*Acremonium*	sp.	4H1-S3-P7-3			
UBOCC-A-214042	*Rhodotorula*	*mucilaginosa*	5H1-P0-P7-1	KM222268		KM222342
UBOCC-A-114082	*Exophiala*	sp.	5H1-P0-P7-2	KM222269	KM232464	
UBOCC-A-114083	*Acremonium*	sp.	5H1-P0-P7-3	KM222270	KM232465	
UBOCC-A-214043	*Rhodotorula*	*mucilaginosa*	5H1-P3-P7-1	KM222273		KM222343

UBOCC-A-114084	*Phialophora*	sp.	5H1-P3-P7-2	KM222274	KM232467	
UBOCC-A-114085	*Exophiala*	sp.	5H1-P3-P7-3	KM222275	KM232468	
UBOCC-A-214044	*Rhodotorula*	*mucilaginosa*	5H1-S0-P7-1	KM222277		KM222344
UBOCC-A-214045	*Rhodotorula*	*mucilaginosa*	15H4-P0-P7-1	KM222296		KM222345
UBOCC-A-214046	*Rhodotorula*	*mucilaginosa*	48X3-P0-P7-1			
UBOCC-A-114086	*Fusarium*	*solani*	48X3-P0-P7-2	KM222302	KM232480	
UBOCC-A-214047	*Rhodotorula*	*mucilaginosa*	48X3-S0-P7-1	KM222304		KM222346
UBOCC-A-214048	*Rhodotorula*	*mucilaginosa*	3H5-P3-P8-1			
UBOCC-A-214049	*Rhodotorula*	*mucilaginosa*	3H5-M3-P8-1	KM222242		KM222347
UBOCC-A-214050	*Rhodotorula*	*mucilaginosa*	5H1-M0-P8-1			
UBOCC-A-114087	*Penicillium*	sp.	5H1-M0-P8-2	KM222262	KM232457	
UBOCC-A-114088	*Acremonium*	sp.	5H1-M3-P8-1	KM222264	KM232458	
UBOCC-A-214051	*Rhodotorula*	*mucilaginosa*	5H3-P3-P8-1	KM222287		KM222348
UBOCC-A-214052	*Rhodotorula*	*mucilaginosa*	5H3-M0-P8-1			
UBOCC-A-114089	*Acremonium*	sp.	5H3-M0-P8-2			
UBOCC-A-214053	*Rhodotorula*	*mucilaginosa*	5H3-M3-P8-1	KM222281		KM222349
UBOCC-A-214054	*Rhodotorula*	*mucilaginosa*	48X3-S3-P8	KM222305		KM222313
UBOCC-A-114090	*Rhodotorula*	*mucilaginosa*	88X1-P0-P8-1			
UBOCC-A-114091	*Rhodotorula*	*mucilaginosa*	88X1-P3-P8-1			
UBOCC-A-214055	*Rhodotorula*	*mucilaginosa*	88X1-P3-P8-2	KM222308		KM222312
UBOCC-A-114092	*Rhodotorula*	*mucilaginosa*	88X1-S3-P8-1			
UBOCC-A-114093	*Rhodotorula*	*mucilaginosa*	88X1-M3-P8-1			
UBOCC-A-114094	*Cladosporium*	sp.	CB1	KM222206	KM232484	
UBOCC-A-114095	*Bjerkandera*	sp.	CB2	KM222207	KM232485	
UBOCC-A-114096	*Fusarium*	sp.	CB3	KM222208	KM232486	
UBOCC-A-114097	*Penicillium*	*brevicompactum*	CB4			
UBOCC-A-114098	*Penicillium*	*brevicompactum*	CB5			
UBOCC-A-114099	*Penicillium*	*brevicompactum*	CB6	KM222209	KM232487	
UBOCC-A-114100	*Penicillium*	*brevicompactum*	CB7			
UBOCC-A-114101	*Penicillium*	*brevicompactum*	CB8			
UBOCC-A-114102	*Penicillium*	*brevicompactum*	CB9	KM222210	KM232488	
UBOCC-A-114103	*Penicillium*	*brevicompactum*	CB10	KM222211	KM232489	

UBOCC-A-114104	*Penicillium*	*chrysogenum*	CB11		
UBOCC-A-114105	*Bullera*	*unica*	CB12	KM222212	KM232490
UBOCC-A-114106	*Penicillium*	sp.	CB13	KM222213	KM232491
UBOCC-A-114107	*Microascus*	*cirrosus*	CB14	KM222204	KM232492
UBOCC-A-114108	*Microascus*	*cirrosus*	CB15		
UBOCC-A-114109	*Penicillium*	sp.	CB16	KM222214	KM232493
UBOCC-A-114110	*Penicillium*	*chrysogenum*	CB17	KM222215	KM232494
UBOCC-A-114111	*Penicillium*	*chrysogenum*	CB18	KM222216	KM232495
UBOCC-A-114112	*Cladophialophora*	*bantiana*	CB19	KM222217	KM232496
UBOCC-A-114113	*Cladosporium*	sp.	CB20	KM222205	KM232597
UBOCC-A-114114	*Agaricomycetes*	sp.	CB21	KM222218	KM232498
UBOCC-A-114115	*Agaricomycetes*	sp.	CB22		
UBOCC-A-114116	*Agaricomycetes*	sp.	CB23		
UBOCC-A-114117	*Penicillium*	*chrysogenum*	CB24	KM222219	KM232499
UBOCC-A-114118	*Cladophialophora*	*bantiana*	CB25	KM222220	KM232500
UBOCC-A-114119	*Cladophialophora*	*bantiana*	CB26	KM222221	KM232501
UBOCC-A-114120	*Penicillium*	sp.	CB27		
UBOCC-A-114121	*Cordyceps*	*confragosa*	CB28	KM222222	KM232502
UBOCC-A-114122	*Penicillium*	sp.	CB29		
UBOCC-A-114123	*Penicillium*	*chrysogenum*	CB30	KM222223	KM232503
UBOCC-A-114124	*Penicillium*	*chrysogenum*	CB31		
UBOCC-A-114125	*Penicillium*	*chrysogenum*	CB32		
UBOCC-A-114126	*Eurotium*	*herbariorum*	CB33	KM222224	KM232504
UBOCC-A-114127	*Eurotium*	*herbariorum*	CB34		
UBOCC-A-114128	*Sistotrema*	*brinkmannii*	CB35	KM222225	KM232505
UBOCC-A-114129	*Oidiodendron*	*griseum*	CB36	KM222226	KM232506
UBOCC-A-114130	*Sistotrema*	*brinkmannii*	CB37	KM222227	KM232507

816

Table S2. Relative occurrence of PKSI, PKSIII, NRPS, Hybrids and TPS genes in filamentous fungi (A) and yeasts (B)

A	FILAMENTOUS FUNGI				
Depth (mbsf)	PKSI	PKSIII	NRPS	Hybrids	TPS
4	0,82	0,73	0,73	0,64	0,36
12	1	0,75	0,5	0,75	0,25
21	1	0,67	0,5	0,5	0
25	1	0,59	0,94	0,82	0,47
34	0,95	0,41	0,77	0,73	0,41
37	1	1	0,67	0,83	0
137	0,88	0,5	0,88	0,63	0,75
403	1	0,14	0,86	0,71	0,14
765	0,71	0,29	0,43	0,43	0,43

B	YEASTS				
Depth (mbsf)	PKSI	PKSIII	NRPS	Hybrids	TPS
4	0,4	0,2	1	0	0,4
12	1	0,33	1	0	0,67
21	0,5	0,33	0,5	0	0,33
25	0,5	0,38	0,63	0	0,5
34	1	0,56	0,78	0	0,67
37	0,78	0,44	0,78	0	0,78
137	0,6	0,2	0,2	0	0,4
403	0,75	0,25	1	0	1
765	0,5	0,25	0,75	0	0,5

Etude 4 : Insights into fungal metabolisms in deep subseafloor sediments.

En préparation

Rédou V., Pachiadaki M.G., Edgcomb V., Barbier G.and Burgaud G.

Les sédiments marins représentent le principal réservoir de carbone organique sur Terre (Fry *et al.,* 2008) et, à cet effet, repésente un écosystème complexe en terme de diversité et d'interactions biogéochimiques. Bien qu'il soit clairement établi que les populations microbiennes peuvent persister sous la surface du plancher océanique, leurs fonctions et leurs interactions avec l'environnement sont encore mal connues. Une meilleure compréhension de l'activité de la biosphère profonde est essentielle afin de définir son rôle dans les cycles biogéochimiques globaux.

Il est maintenant important d'amorcer des travaux de métatranscriptomique servant à identifier clairement les mécanismes adaptatifs mis en jeu par les micro-organismes pour répondre aux contraintes environnementales inhérentes à cet écosystème extrême. La première étude métatranscriptomique effectuée sur des échantillons de sédiments marins a permis de mettre en évidence la capacité de communautés microbiennes procaryotiques et fongiques à se reproduire par division cellulaire. Capables de dégrader une variété de substrats organiques comme les acides aminés, les lipides ou les glucides, les micro-organismes de la biosphère profonde ont donc un rôle écologique dans les grands cycles géochimiques (Orsi *et al,.* 2013b).

L'objectif de cette étude était de décrire et analyser le métatranscriptome eucaryote issu de deux échantillons du bassin de Canterbury, en réplicat, prélevés à respectivement 5,60 et 345,50 mètres sous la surface du plancher océanique, en ciblant spécifiquement les queues poly-A des ARNm.

La préparation des librairies d'ADNc, effectuée à l'université du Delaware (USA), a généré des librairies de mauvaise qualité. En effet, nous avons obtenu des profils ADN de

faible poids moléculaire représentant une faible diversité au sein des échantillons. Bien que le même protocole ait été suivi pour tous les échantillons, un seul a présenté un nombre de reads correct. Dans ce contexte, nous avons choisi d'analyser uniquement cet échantillon. Les résultats préliminaires révèlent des activités métaboliques fongiques principalement liées à la croissance, à l'adaptation aux conditions environnementales *in situ* et aux interactions entre communautés microbiennes.

Ces données fournissent la preuve directe que les communautés fongiques sont métaboliquement actives à des profondeurs record dans les sédiments marins profonds, que ces communautés intéragissent avec les commautés procaryotiques et mettent en place des mécanismes pour résister aux contraintes environnementales.

In preparation

Insights into fungal metabolisms in deep subseafloor sediments

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Running title

Eukaryotic metabolisms in the deep biosphere.

Abstract

The deep biosphere is colonized by a large prokaryotic and eukaryotic diversity but microbial metabolisms in the deep subsurface are poorly understood. Low nutrient availability and energetic constraints shape the physiological status and growth potential of deeply buried microbial communities. Currently, it is not totally clear whether cells in subsurface samples are alive, growing, surviving, dormant, or dead fossils. In this study, we describe a subseafloor metatranscriptome from the Canterbury basin targeting eukaryotic mRNA. Among the 11,389 retrieved contigs, we revealed fungal transcripts associated with growth, resistance to subseafloor environmental constraints and interaction with the deep biosphere. These data provide evidence that fungal communities are metabolically active at record depths in deep sediments and may play an important role in biogeochemical cycles.

Keywords

Subsurface sediments, Canterbury basin, mRNA, Poly-A, eukaryotic metatranscriptome, gene ontology

Introduction

The deep subsurface harbors a large fraction of Earth's living biomass (Kallmeyer *et al*., 2012). Studies of ribosomal (SSU rRNA) and messenger RNA suggest that the deep biosphere, represented by members of the three domains of life (*Archaea*, *Bacteria* and *Eukarya)*, includes metabolically active communities (Edgcomb *et al*., 2011; Orsi *et al.,* 2013a, 2013b). Eukaryotic SSU rRNA sequences detected in marine sediments are affiliated to already-described lineages in the eukaryotic domain. These microeukaryotes may adapt to *in situ* conditions and thus may play important roles in the deep biosphere through the utilization and recycling of nutrients. Indeed, a DNA-based study revealed that the fungi appeared to be the most frequently detected eukaryotes in the Canterbury Basin up to 1740 meters below the sea floor (Ciobanu *et al*., 2014). Eukaryotic rRNA-based studies conducted in Peru margin and Peru trench (Edgcomb *et al*., 2011; Orsi *et al.,* 2013a) are in agreement

with this findings and indicate that fungi are metabolically active. This clearly suggests that fungi may be specifically adapted to the deep subsurface *in situ* conditions and may play important roles in biogeochemical cycles (Edgcomb *et al*., 2011, Orsi *et al*., 2013a).

Recent DNA-based studies have extended the deep biosphere with the occurrence of persisters down to 1922 mbsf (Ciobanu *et al.,* 2014). The application of transcriptomic in deep biosphere samples demonstrated the occurrence of microbial activity and growth in the subsurface, including fungal activity (Orsi *et al*., 2013b). Indeed, fungi in deep sediments appeared to be involved in carbohydrate, amino acid and lipid metabolism, indicating a potentially significant role in organic carbon turnover in subseafloor sediments.

To extend the investigation of eukaryotic activities in subsurface sediments, we investigated eukaryotic functions from a 345.50 mbsf depth sample collected in the Canterbury basin, off the coast of New Zealand (IODD, Leg 317) using a eukaryotic-targeted method to extract, sequence and analyze fungal mRNA. The Canterbury basin was analyzed because a wealth of microbial data exists for this site that exhibits bacterial, archaeal and microeukaryotic communities.

Materials and Methods

1. Site description and sediment sampling

Sediment samples were collected at the Canterbury Basin, on the eastern margin of the South Island of New Zealand, during IODP leg 317 expedition (JOIDES Resolution). Sediment core was drilled at Site U1352 (44°56’26.62’’S; 172°1’36.30’’E) at 344 m water depth and a total cored sediment depth of 1927.5 mbsf, spanning the Holocene to late Eocene periods. The core lithology was described in Ciobanu *et al*., (2014).

2. RNA extraction, purification and amplification

RNA extractions were performed from 1 sample collected at 345.50 mbsf (sample name 42X3). In order to avoid contamination, all manipulations were carried out in a PCR hood. An extraction blank was also carried through the entire procedure to control for contamination.

80 RNA was extracted from 16 grams of uncontaminated frozen samples (-80°C) with the RNA
81 PowerSoil® Total RNA Isolation Kit (#12866-25, MoBio Laboratories), following the
82 manufacturer's instructions. At the final step, the RNA was eluted with 100 µl of DES
83 solution. To remove potential DNA contamination, RNA extracts were treated using the
84 Turbo DNA-free kit (Life Technologies). To purify the RNA extracts, the MEGAclear RNA
85 purification kit (Life Technologies) was used according the manufacturer's instructions.
86 Removal of contaminating DNA in RNA extracts was confirmed by the absence of visible
87 amplification of small subunit ribosomal RNA genes after 40 cycles of PCR using the RNA
88 extracts as template. Total RNA was used as template for cDNA amplification using the
89 Ovation 3'-DGE System (NuGEN technologies) to obtain an enriched eukaryotic
90 transcriptome. Double stranded cDNA was cleaned with the DNA Clean & Concentrator kit
91 (Zymo Research) using a modified protocol provided by NuGen
92 (https://www.biolynx.ca/pdf/admin/nu_threedgeuserguide.pdf). The quantity of the amplified
93 cDNA was checked on Fluorometer (Qubit 2.0, Life Technologies).

94

95 **3. Illumina library preparation and sequencing**

96 Library preparation and paired-end 2x150 Illumina Hi-Seq sequencing was performed at the
97 University of Delaware Sequencing and Genotyping Center (Delaware Biotechnology
98 Institute).

99

100 **4. Eukaryotic transcriptome analyses**

101 The forward and reverse reads were filtered using Trimmomatic (Bolger *et al.,* 2014), which
102 performs a "sliding window" trimming, cutting once the average quality within the window (8
103 nt) falls below a threshold (12). The length of the trimmed sequences was set to be at least 50
104 nt. The trimmed reads surviving quality control were uploaded onto the Data Intensive
105 Academic Grid (DIAG, http://diagcomputing.org/) and were assembled into contigs using
106 Trinity (Grabherr *et al*., 2011). Blast x analysis were run through the Blast2Go platform
107 (Conesa *et al*., 2005) using an e-value of 10^{-3} and fungal taxonomy filter (fungi nr subset
108 database). Contigs were then mapped and annotated to clusters of gene ontologies (GO) using
109 an e-value of 10^{-6} and fungal taxonomy filter (fungi nr subset database).

110

111 **Results and discussion**

112 A *de novo* assembly using all 5,250,985 reads was performed and generated 11,389 contigs
113 with an average length of 1,705 pb. 930 sequences that were assigned to fungal taxa. As a first
114 conclusion, fungi appeared metabolically active in the deep subseafloor albeit relative
115 abundances of fungal transcripts seem weak.

116 Contigs were assigned to a wide range of gene ontology categories. Although many of the
117 contigs did not produce significant BLAST hits, a wide diversity of fungal transcripts
118 involved in different functions was represented in our dataset (Fig. 1). Few 'omics' analyses
119 have been dedicated to the study of the subseafloor biosphere: (i) two metagenomes (Biddle *et*
120 *al.,* 2008; 2011), (ii) three single-cell genomic analyses (Kaster *et al.,* 2014; Lloyd *et al*.,
121 2013; Wasmund *et al*., 2013) and (iii) one metatranscriptomic survey (Orsi *et al*., 2013b).
122 With only those 6 extensive studies focusing on the deep biosphere, debates still occur
123 regarding whether cells are alive and metabolically active (Jørgensen, 2011) or mostly
124 microbial zombies (Colwell & D'Hondt, 2013). Our results provide further support for the
125 notion of fungal activity in the deep subseafloor at record depths, extending the previously
126 known depth limits of eukaryotic functions from 159 to 345.50 mbsf.

127 Gene expression from fungal communities was mainly assigned to metabolic and cellular
128 processes, cell and membrane functions, and catalytic activity (Figure 1). Our aim was then to
129 search for specific fungal functions in the gene expression profile to answer the following
130 ecological questions: (i) Are fungi able to grow and sporulate in the deep subseafloor ?, (ii)
131 How are fungi able to cope with *in situ* environmental constraints ? and (iii) Are fungi able to
132 interact with other members of the microbial community?

133 Several DNA-based analyses have revealed the presence of fungal communities in the deep
134 subseafloor (Edgcomb *et al*., 2011; Orsi *et al*., 2013a, 2013b; Ciobanu *et al.,* 2014), but only
135 two studies provide evidence that part of these communities were active. Edgcomb *et al.*
136 (2011) and Orsi *et al.* (2013b) have respectively used an rRNA and mRNA-based analysis to
137 report their activity and functions in the deep subseafloor. Orsi *et al.* (2013b) reported fungal
138 cell-division transcripts, indicating a diversity of actively dividing cells, but also fungal
139 transcripts involved in carbohydrate, amino acid and lipid metabolism, suggesting that fungi

140 have a role in organic carbon turnover in subseafloor sediment (Orsi *et al.,* 2013b). In our
141 metatranscriptome sample, cell-division transcripts (cytokinesis) were also retrieved
142 indicating still actively dividing fungal cells at 345.50 mbsf (Table 1). Interestingly, fungal
143 transcripts involved in mycelium development, ergosterol biosynthesis, filamentous growth
144 and hyphal growth were also detected. It clearly indicates an effective fungal growth in deeply
145 buried sediment. Spore germination and conidium formation transcripts strongly indicate an
146 effective fungal sporulation. Detection of transcripts involved in mycelium formation, fungal
147 cell membrane (ergosterol biosynthesis) and sporulation are direct evidence for active fungal
148 growth and sporulation in the deep biosphere and clearly indicate that physical and
149 geochemical constraints at 345.50 mbsf are not a bottleneck for fungal life.

150 Nine per cent of fungal transcripts were involved in carbohydrate, amino acid and lipid
151 metabolism (Fig. 2). Fungal expression of transcripts coding mainly for hydrolases, indicates
152 that they degrade a variety of organic substrates in deep sub-seafloor sediment (Fig. 3). These
153 results are consistent with previous metatranscriptomic work (Orsi *et al*., 2013b) and suggest
154 that the active fungal communities have a role in biogeochemical cycles with organic carbon
155 turnover in subseafloor sediment.

156 Nitrate reduction has been reported to occur in the deep subseafloor with *Alphaproteobacteria*
157 and *Betaproteobacteria* as predominant actors (Orsi *et al.,* 2013b). Authors argue that the
158 resulting nitrite was probably reduced by fungi, *Gammaproteobacteria* and *Firmicutes*.
159 Transcripts involved in nitrite reduction were reported in our dataset, supporting the previous
160 idea of syntrophic interactions in the deep subseafloor. Such results are also supported by
161 previous studies revealing the potentially active role of fungi in denitrification in marine
162 sediments (Jebaraj *et al.,* 2010; Mouton *et al.,* 2012).

163 Several fungal transcripts were involved in stress tolerance, *i.e.* starvation, heat, hypoxia,
164 oxidative stress and DNA damage. Such transcripts indicate that stress responses occur and
165 represent mechanisms by which fungi are able to cope with different kinds of stress. As DNA
166 degradation could be seen as a major limitation to long-term dormancy, the occurrence of
167 DNA repair transcripts, as suggested by Jørgensen *et al.* (2011) and Orsi *et al.* (2013b),
168 supports the idea that dormancy may not be a dominant survival strategy in the deep
169 biosphere, both for prokaryotes and fungi. Indeed, DNA repair may represent an adaptive
170 mechanism to handle the slow degradation of DNA over geological timescales.

171 Transcripts involved in ergosterol biosynthesis have been detected (see above). Ergosterol is
172 an important component of fungal membrane but is also known to have a protection role of
173 fungal cell membrane (Fernandes, 2004). Indeed, it has been shown that *S. cerevisiae* is able
174 to modify its membrane composition in order to tolerate high hydrostatic pressure conditions
175 in increasing the proportion of unsaturated fatty acids and ergosterol (Simonato *et al*., 2006).
176 This adaptive strategy is designed to increase membrane fluidity to counteract the effect of
177 hydrostatic pressure and thus help maintain the functionality of the cell membranes in deep
178 ecosystems. The occurrence of ergosterol biosynthesis transcripts may be another mechanism
179 by which deep subseafloor fungi are able to cope with elevated hydrostatic and lithostatic
180 pressures in the deep biosphere.

181 Fungal expression of secondary metabolite biosynthesis was detected with asperthecin,
182 penicillin, emericellamide metabolic process transcripts (Table 1). Fungal expression of
183 siderophores was also detected (Table 1). Siderophores could have different activities apart
184 from transport of metals, such as antioxidant and antibiotic action (Sebat *et al.,* 2001).
185 Microbial expression of defense mechanisms suggests that fungi are able to interact and
186 compete with prokaryotic communities occurring in the deep subseafloor. The detection of
187 fungal transcripts involved in secondary metabolite biosynthetic process, as nonribosomal
188 peptide biosynthetic process, also supports such a conclusion. A previous study has provided
189 direct evidence that fungal isolates from deep subseafloor sediments harbor genes involved in
190 secondary metabolite biosynthesis pathways (Rédou *et al.,* submitted). Both studies warrant
191 further investigation to search for potentially novel and useful secondary metabolites
192 produced by deep subseafloor microorganisms. We hypothesize that the deep biosphere
193 represents an interesting ecosystem to help tackle the innovation crisis related to the
194 development of new molecular entities in pharmaceutical industry (Kling *et al.,* 2014).

195 Many subsurface bacterial isolates host prophages (Engelhardt *et al*., 2011, 2014), with nearly
196 half of the bacterial isolates tested from a deep-sea sediment core harboring prophage. In
197 agreement with such studies on prokaryotes, transcripts involved in viral life cycle and virion
198 assembly are present in our study. The ubiquity of lysogeny could have interesting
199 implications for cell survival in this ecosystem where energy sources are limited and the
200 microorganisms are subjected to strong selection pressure (Anderson *et al*., 2013). Some
201 lysogenic phages can induce gene silencing of host metabolism and limit the metabolic

processes of the host when the conditions are not favorable (Paul, 2008). This characteristic of lysogenic phages could be particularly useful in deep environments where access to energy sources is limited. If the impact of viruses in nutrient recycling and production of labile organic matter is found in surface sediments (Danovaro *et al*., 2008), there is no comparable data available at the deep biosphere. The ubiquity of lysogeny in *Archaea* and *Bacteria* stated by Anderson *et al.* (2011) seems to be also true for fungi. The understanding of viral distribution and ecological role in deep-sea sediments is still poorly known and raises many questions about the origin of virus and their impact on microbial communities in the environment (Middelboe *et al*., 2011).

Conclusion

Our metatranscriptomic analysis enables a refined view of the deep biosphere with the occurrence, activity and metabolic functions of fungal communities. Our data allow us to extend evidence for fungal gene expression in the deep subseafloor from 159 to 345.50 mbsf and provide insights regarding fungal growth, resistance, roles in biogeochemical cycles and interactions with the deep biosphere.

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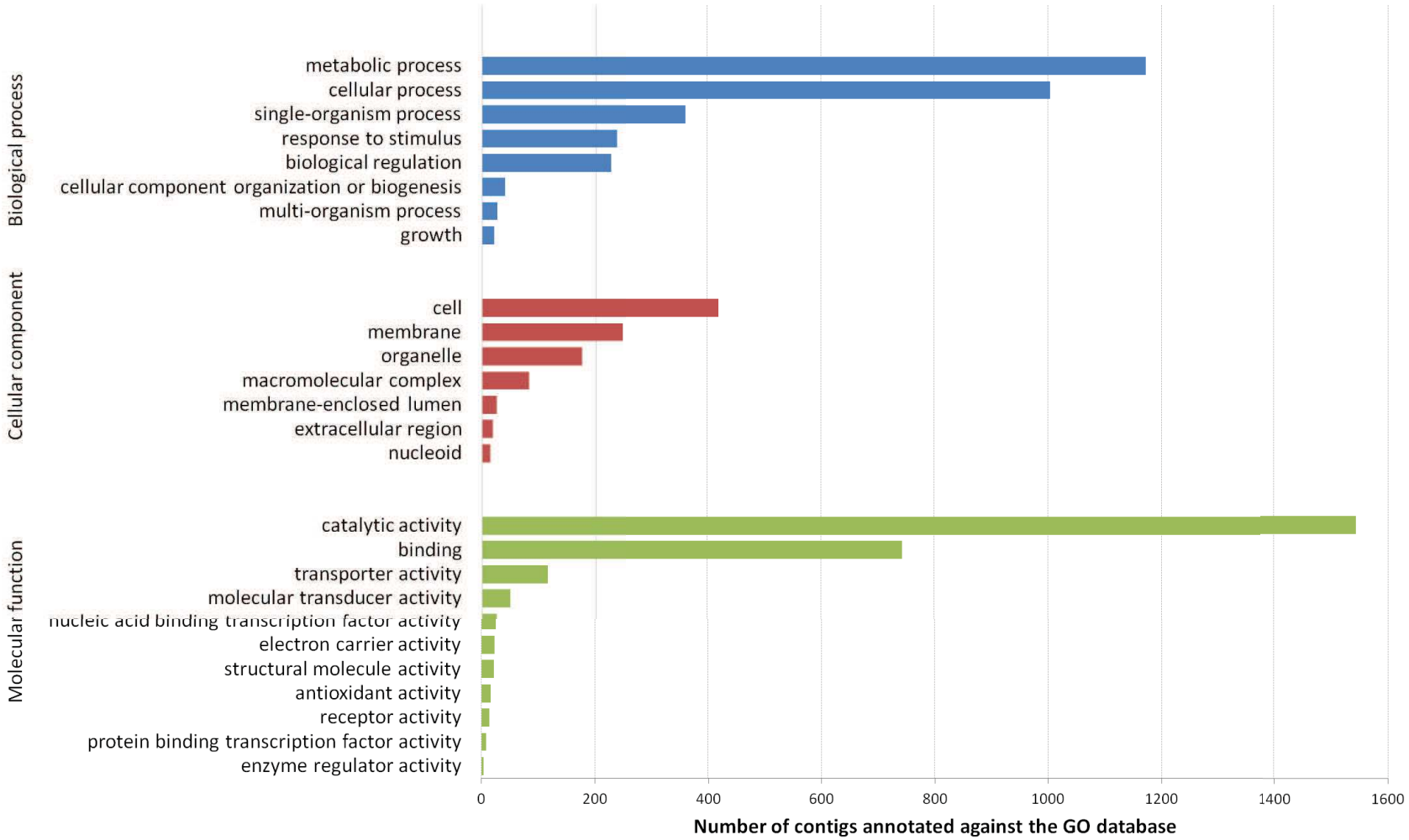
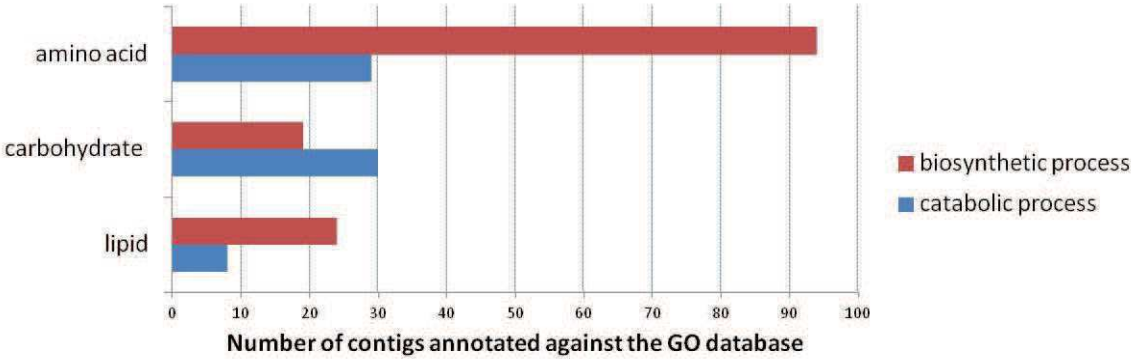
295 **Figure 1:** Gene ontology distribution of fungal contigs.



296

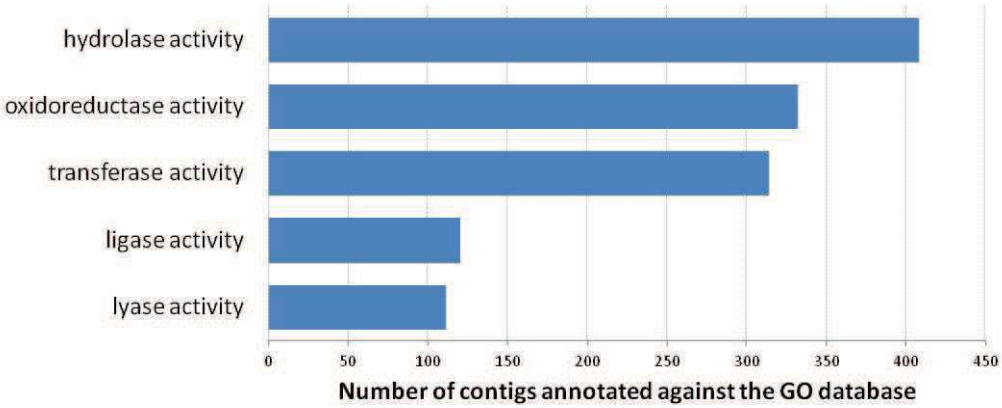
297 **Figure 2**: Fungal metabolic process.



298

299

300 **Figure 3:** Fungal catalytic activity.



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302

303

Table 1 : Fungal functions occurring in subseafloor.

Growth	Resistance	Interaction
cytokinetic process	DNA repair	nonribosomal peptide biosynthetic process
ergosterol biosynthetic process	response to starvation	asperthecin metabolic process
spore germination	response to hypoxia	penicillin metabolic process
conidium formation	response to oxidative stress	emericellamide metabolic process
mycelium development	response to heat	
filamentous growth		
hyphal growth		

304

Chapitre III : CONCLUSIONS ET PERSPECTIVES

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Les champignons ont la capacité de coloniser différents types d'habitats, qu'ils soient terrestres, aquatiques dulçaquicoles ou marins. Largement étudiés en milieu terrestre, les champignons sont des acteurs clés des cycles biogéochimiques de par leur implication (i) dans la dégradation de la matière organique en tant que saprophyte et (ii) dans la régulation des communautés en tant que parasites. En milieu marin, la compréhension du rôle écologique des champignons n'en est qu'à ses débuts. En effet, jusqu'à présent quelques études moléculaires ont révélé que les champignons occupaient une place importante, parmi les micro-eucaryotes, en termes d'abondance, dans les environnements marins mais, à ce jour, peu de représentants cultivables ont été déposés dans des collections de cultures. Dans ce contexte, l'objectif de ce travail de thèse était de mettre en évidence la diversité fongique de subsurface par approche moléculaire haut-débit et par approche culturale afin de proposer des hypothèses sur leur(s) adaptation(s) aux conditions environnementales extrêmes et leur(s) implication écologique(s) au sein de la biosphère profonde.

3.1. Diversité fongique en écosystème sédimentaire marin profond

Les études de diversité fongique par approche moléculaire ciblent généralement les séquences correspondant à la petite sous-unité de l'ARNr (18S) ou à l'ITS. La variabilité des régions ITS offre une meilleure assignation taxonomique que l'ARNr 18S et une précision au niveau de l'espèce. Cependant, l'ITS présente une faible résolution phylogénétique contrairement à l'ARNr 18S et il est donc difficile d'établir des relations de parenté entre les espèces. Par conséquent, lors de ce travail de thèse nous avons choisi d'utiliser à la fois l'ARNr 18S et l'ITS afin de préciser la position phylogénétique et valider l'assignation taxonomique des séquences étudiées (Chapitres 1 et 2). Au terme de ces analyses de diversité, en utilisant des techniques de séquençage haut-débit, nous avons révélé une diversité de micro-organismes eucaryotes majoritairement fongiques, jusqu'à présent insoupçonnée en écosystème sédimentaire marin profond. En effet, nos données fournissent la preuve que les communautés fongiques sont présentes, jusqu'à des profondeurs records au sein de la subsurface du bassin de Canterbury. Nous avons mis en évidence la présence de 27 OTUs fongiques différents, appartenant aux phyla des *Ascomycota* et *Basidiomycota,* de 346 à 1740

mètres. De façon intéressante, la majorité des taxons identifiés ont auparavant été détectés dans les sédiments marins lors de précédentes études (Tableau 1) ou plus largement dans l'environnement marin. L'absence de représentants de l'ancien phylum des Zygomycètes est cohérente avec les études précédentes. En effet, à l'heure actuelle aucun Zygomycète n'a été détecté par approche moléculaire dans les environnements profonds ce qui suggère que ces champignons sont très rares ou inexistants dans ces écosystèmes singuliers. Cependant ces résultats sont à prendre avec précaution car il se pourrait que les amorces utilisées n'amplifient pas les Zygomycètes des environnements profonds (Nagano et Nagahama, 2012). En revanche, l'absence de signatures de Chytridiomycètes dans notre étude suggère qu'ils seraient absents ou très peu abondants au niveau du bassin de Canterbury. La majorité des chytrides sont isolés en milieu aquatique mais leur présence dans les environnements marins a également été reportée (Nagahama *et al.*, 2011). En effet, d'anciennes lignées évolutives du phylum des *Chytridiomycota* ont été révélées dans les écosystèmes hydrothermaux profonds (Le Calvez *et al*., 2009). L'étude de Nagahama *et al.* (2011) a également mis en évidence que les champignons basaux pouvaient occuper une grande partie des clones fongiques au niveau de zones de suintements froids, riche en méthane. Les chytrides sont habituellement retrouvés en tant que saprophytes ou parasites (Gleason *et al*., 2008). Ainsi, l'absence d'organismes supérieurs dans les sédiments pourrait être à l'origine de leur absence dans cet écosystème. La majorité des signatures moléculaires de *Chytridiomycota* et de champignons basaux, détectée dans les environnements marins, représente de nouveaux phylotypes. Ces nouveaux champignons pourraient avoir des caractéristiques écologiques et physiologiques uniques ce qui expliquerait pourquoi aucun représentant n'ait encore été obtenu par méthode culturale (Nagano et Nagahama, 2012).

Tableau 1 : Signatures fongiques mises en évidence dans les sédiments marins par approche moléculaire

	Singh *et al*., 2011 deep-sea sediment SSU/ITS	Singh *et al.*, 2012 deep-sea sediment SSU/ITS	Nagano *et al*., 2010 deep-sea sediment ITS	Xu *et al*., 2014 deep-sea sediment ITS	Edgcomb *et al*., 2011 subsurface sediment SSU	Orsi *et al*., 2013a subsurface sediment SSU	Rédou *et al*., unpublished subsurface sediment SSU/ITS
Acaulospora			*				
Acidomyces						*	
Alternaria						*	
Antrodia						*	
Aphyllophorales							
Apioplagiostoma						*	
Aspergillus	*	*	*	*			
Aureobasidium				*			
Auricularia				*			
Batcheloromyces							*
Camarops						*	
Candida	*		*	*		*	
Capnodium	*						
Cerrena		*					
Chaetomium				*			
Cladosporium				*			
Cordycep						*	
Corynespora		*					
Crinipellis						*	
Cryptococcus			*	*	*	*	*

	Singh *et al*., 2011 deep-sea sediment SSU/ITS	**Singh *et al.*, 2012 deep-sea sediment SSU/ITS**	**Nagano *et al*., 2010 deep-sea sediment ITS**	**Xu *et al*., 2014 deep-sea sediment ITS**	**Edgcomb *et al*., 2011 subsurface sediment SSU**	**Orsi *et al*., 2013a subsurface sediment SSU**	**Rédou *et al*., unpublished subsurface sediment SSU/ITS**
Cryptosporella						*	
Cyberlindnera						*	*
Debaryomyces	*	*					
Dioszegia						*	
Discula						*	
Diversispora						*	
Doassansia						*	
Elmerina							*
Emericella							
Entoloma						*	
Erythrobasidium						*	
Exophiala				*			*
Filobasidium						*	*
Fusarium				*			*
Galactomyces							*
Geopyxis						*	
Gibberella		*					
Gloeotinia				*			
Glomerella			*				
Glomus						*	
Glyphium						*	
Golovinomyces							

	Singh *et al.*, 2011 deep-sea sediment SSU/ITS	**Singh *et al.*, 2012 deep-sea sediment SSU/ITS**	**Nagano *et al.*, 2010 deep-sea sediment ITS**	**Xu *et al.*, 2014 deep-sea sediment ITS**	**Edgcomb *et al.*, 2011 subsurface sediment SSU**	**Orsi *et al.*, 2013a subsurface sediment SSU**	**Rédou *et al.*, unpublished subsurface sediment SSU/ITS**
Hannaella						*	
Helicogloea						*	
Hortaea	*	*					
Hydropus						*	
Hyphodontia						*	
Knufia						*	
Lecanicillium				*			
Lentinula						*	
Leptosphaerulina							*
Leucosporidium						*	*
Malazessia	*	*		*	*		*
Metschnikowia			*				
Meyerozyma				*			*
Mycena						*	
Nectria	*						
Nematoctonus							
Neurospora		*				*	
Nodulisporium	*						
Paecilomyces				*			
Penicillium			*	*			*
Periconia				*			
Peyronellaea						*	

	Singh *et al.*, 2011 deep-sea sediment SSU/ITS	**Singh *et al.*, 2012 deep-sea sediment SSU/ITS**	**Nagano *et al.*, 2010 deep-sea sediment ITS**	**Xu *et al.*, 2014 deep-sea sediment ITS**	**Edgcomb *et al.*, 2011 subsurface sediment SSU**	**Orsi *et al.*, 2013a subsurface sediment SSU**	**Rédou *et al.*, unpublished subsurface sediment SSU/ITS**
Phialosimplex		*					
Phoma	*						
Phruensis						*	
Pichia	*						
Pleurostomophora							*
Pleurotus				*			
Powellomyces						*	
Pycnoporus		*					
Ramichloridium				*			
Resinicium		*					
Rhinocladiella							*
Rhizoctonia						*	
Rhizophlyctis			*				
Rhodosporidium	*					*	*
Rhodotorula	*	*	*			*	*
Saccharomyces		*					
Sagenomella	*						
Schizophyllum		*					
Simplicillium				*			
Sordaria						*	
Sporobolomyces						*	
Steccherinum						*	

	Singh *et al*., 2011 **deep-sea sediment** **SSU/ITS**	**Singh *et al.*, 2012** **deep-sea sediment** **SSU/ITS**	**Nagano *et al*., 2010** **deep-sea sediment** **ITS**	**Xu *et al*., 2014** **deep-sea sediment** **ITS**	**Edgcomb *et al*., 2011** **subsurface sediment** **SSU**	**Orsi *et al*., 2013a** **subsurface sediment** **SSU**	**Rédou *et al*., unpublished** **subsurface sediment** **SSU/ITS**
Stenella	*	*					
Stereum						*	
Sterigmatomyces		*				*	
Trametes				*			
Tremella							*
Trichoderma							*
Trichosporon	*	*	*	*	*		*
Typhula							
Ulospora	*						
Wallemia	*						*
Xylaria				*			

En complément de l'approche moléculaire, nous avons mis en place une approche culturale afin de caractériser les communautés fongiques cultivables des sédiments marins (Chapitre 3). Afin de se rapprocher des conditions *in situ*, nous avons choisi d'utiliser la pression hydrostatique pour améliorer l'efficacité de culture. En effet, les pressions hydrostatiques et lithostatiques élevées représentent un paramètre majeur pour définir la biosphère profonde. L'approche culturale utilisée nous a révélé une toute autre image des communautés fongiques des sédiments marins profonds. En effet, parmi les 21 genres identifiés en culture, 16 n'ont pas été mis en évidence par approche moléculaire (Tableau 2). Ces résultats soulignent que les techniques moléculaires, même à haut débit, ne permettent pas de révéler l'ensemble des communautés ciblées et que l'approche culture-dépendante reste nécessaire pour obtenir une vision la plus exhaustive possible des communautés présentes. Bien que les méthodes dites culture-indépendantes permettent de mettre en évidence de nombreux taxons fongiques (Nagano *et al*., 2010; Jones *et al*., 2011a; Nagahama *et al*., 2011) et donc de traduire au mieux la réalité de la diversité dans l'écosystème sédimentaire, il faut cependant noter que ces méthodes présentent des biais via la PCR, le choix des amorces et l'analyse des données (Anderson *et al*., 2003; Stoeck *et al*., 2006; Bellemain *et al*., 2010; Richards *et al*., 2012).

Les 138 champignons filamenteux de la collection constituée sont majoritairement affiliés aux *Ascomycota* et seulement 13 souches appartiennent au phylum des *Basidiomycota*. A l'inverse, concernant les levures, 45 souches appartiennent aux *Basidiomycota* et 14 souches à celui des *Ascomycota*. La même tendance a précédemment été observée au sein d'une collection d'isolats fongiques issus d'échantillons de sources hydrothermales profondes (Burgaud *et al*., 2009, 2010). Des biais de culture liés aux techniques utilisées peuvent expliquer ces mêmes ratios d'isolement « forme filamenteuse / forme levure ». Cependant, lors de cette thèse, des résultats convergents ont été obtenus par approche culturale et moléculaire, *i.e.* un nombre limité de levures par rapport aux champignons filamenteux et semble valider la dominance de la forme filamenteuse en écosystème sédimentaire.

Tableau 2 : Isolats fongiques mis en évidence dans les sédiments marins par mise en culture

	Mouton *et al*., 2012 deep-sea sediment fungi	Singh *et al*., 2010 deep-sea sediment fungi	Singh *et al*., 2012 deep-sea sediment fungi	Nagahama *et al*., 2001 deep-sea sediment yeasts	Rédou unpublished subsurface sediment fungi
Acremonium	*	*			*
Ascotricha			*		
Aspergillus	*	*	*		*
Bjerkandera					*
Bullera					*
Cerrena			*		
Chaetomium			*		
Cladophialophora					*
Cladosporium		*	*		*
Cordyceps					*
Cryptococcus		*			
Eurotium	*		*		*
Exophiala		*			*
Fusarium					*
Hortaea			*		
Meyerozyma					*
Microascus					*
Nigrospora			*		
Oidiodendron					*
Paecilomyces					*

	Mouton *et al*., 2012 deep-sea sediment fungi	Singh *et al*., 2010 deep-sea sediment fungi	Singh *et al*., 2012 deep-sea sediment fungi	Nagahama *et al*., 2001 deep-sea sediment yeasts	Rédou unpublished subsurface sediment fungi
Paraconiothyrium	*				
Penicillium	*	*	*		*
Phialophora					*
Pleospora			*		
Purpureocillium					*
Rhodosporidium		*			
Rhodotorula	*	*		*	*
Sagenomella		*	*		
Sarcinomyces		*			
Sarocladium					*
Sistotrema					*
Sporidiobolus		*			
Sporobolomyces				*	
Tilletiopsis		*			
Trametes			*		*
Trichoderma	*				
Yarrowia	*				

183 souches, isolées de 4 à 1884 mètres sous la surface du plancher océanique, constituent notre collection. La majorité des isolats ont été mis en évidence dans les premières couches sédimentaire, jusqu'à 37 mbsf. La plus faible diversité observée dans les échantillons les plus profonds pourrait s'expliquer par la réduction de la porosité et de l'accessibilité de la matière organique.

3.2. Dormance ou activité ?

Étonnamment, l'approche moléculaire ciblant l'ARNr ne nous a permis de mettre en évidence qu'un seul OTU fongique contre 13 en utilisant l'ADN. Ces premiers résultats tendent à indiquer que les champignons ne semblent pas être réellement actifs au niveau du bassin Canterbury contrairement à ce que montre de précédentes études, menées au niveau des sédiments de la marge du Pérou (Edgcomb *et al*, 2011; Orsi *et al*, 2013a, 2013b). Cet OTU fongique est affilié à la levure *Malassezia*, souvent mise en évidence en environnement marin (Dawson et Pace, 2002; Edgcomb *et al.*, 2002; Bass *et al.*, 2007; Lai *et al.*, 2007; López-García *et al.,* 2007; Gao *et al.,* 2008; Alexander *et al.*, 2009;. Jebaraj *et al.*, 2010; Edgcomb *et al.*, 2011; Singh *et al.*, 2011). Cependant, aucun isolat de ce genre n'a été obtenu dans notre étude ni dans d'autres études antérieures à partir d'échantillons d'eau de mer ou de sédiments. Nos données basées sur l'ARN suggèrent que la majorité des champignons des sédiments de subsurface du bassin de Canterbury pourraient être en dormance. En effet, dans des conditions ou l'énergie est difficilement accessible, la synthèse microbienne est limitée au maintien de l'intégrité cellulaire (Orcutt *et al.*, 2013). Ainsi, lorsque l'énergie et/ou les nutriments sont peu disponibles, de nombreux micro-organismes entrent en dormance (Price et Sowers, 2004). De récentes études ont montré que la respiration microbienne au sein de la biosphère profonde (D'Hondt *et al.*, 2002, 2004; Røy *et al.*, 2012) est bien plus lente que celle des écosystèmes terrestres (Price et Sowers 2004). Du fait du taux de respiration microbienne extrêmement faible dans les environnements de subsurface, les micro-organismes indigènes sont supposés se reproduire très lentement, voire pas du tout. En effet, le renouvellement de la biomasse de subsurface est extrêmement lent et estimé à plusieurs centaines de milliers d'années (Lomstein *et al.,* 2012). A l'heure actuelle, nous ne savons pas si les micro-organismes de ces environnements souterrains se reproduisent à de faibles taux de

renouvellement ou bien s'ils vivent sans se diviser durant des millions d'années (Colwell et D'Hondt, 2013). Néanmoins, la dormance ne permet pas de stopper complètement la dégradation des composants cellulaires, ni celle de l'ADN (Jørgensen, 2011). La presence de transcrits fongiques impliqués dans la réparation de l'ADN, au cours de notre étude, conforte cette hypothèse que la dormance ne peut pas être une stratégie de survie dominante dans la biosphère profonde. De plus, dans une étude récente de Morono *et al.* (2011) il a été démontré que de nombreux micro-organismes profondément enfouis dans les sédiments marins ont la capacité de croître et de maintenir un potentiel métabolique.

Bien que les champignons constituants notre collection de culture aient une origine terrestre, certains d'entre eux semblent s'être adaptés aux conditions environnementales *in situ* et pourraient avoir un rôle au sein des la biosphère profonde. En se basant sur les données issues de l'étude des communautés procaryotiques, il semblerait que de façon générale les isolats de la biosphère profonde présentent des gammes physiologiques de croissance plus larges que leurs homologues de surface (Sass et Parkes, 2011). De façon cohérente, les souches de notre collection ont été isolées majoritairement avec un enrichissement sous pression hydrostatique élevée. Ainsi, les pressions hydrostatiques et lithostatiques élevées exercées dans les sédiments ne semblent pas être un frein à la germination et la croissance de champignons. Bien qu'aucun champignon piezophile n'ait été mis en évidence à ce jour, il a été mis en évidence que certains champignons seraient capables de développer des mécanismes d'adaptation pour supporter les fortes pressions. Si les analyses physiologiques effectuées lors de notre étude révèlent que la plupart des souches sont halotolérantes, les souches isolées dans les couches sédimentaires les plus profondes semblent être halophiles dans des conditions de températures élevées. Les champignons isolés de la subsurface semblent donc présenter une capacité d'adaptation aux conditions marines au cours de l'enfouissement dans la matrice sédimentaire. De la même manière que les procaryotes, les eucaryotes et plus particulièrement les champignons, pourraient posséder différents modes de vie, de la dormance à l'adaptation en fonction des conditions environnementales.

D'un point de vue génétique, certaines souches affiliées aux *Penicillium*, *Fusarium*, *Paecilomyces* et *Rhodotorula* ont montré des différences génétiques en fonction de la profondeur d'échantillonnage. Récemment, il a été montré que des groupes de gènes du métabolisme secondaire évoluaient rapidement à travers de multiples réarrangements,

duplications ou même la perte de ces gènes (Khaldi *et al*., 2008). Ainsi, il se pourrait que les champignons de subsurface aient perdu certains gènes pour s'adapter à leur environnement. Des analyses physiologiques et génétiques complémentaires devront être effectuées afin de mieux comprendre les adaptations spécifiques mises en œuvre par ces micro-organismes pour survivre dans un écosystème structuré par des contraintes spatiales, physiques et énergétiques.

3.3. Rôle écologique

A l'heure où nous commençons à identifier les tendances en termes d'écologie microbienne procaryotique au sein de la subsurface, la compréhension de l'écologie des communautés virales et eucaryotes n'en est qu'à ses débuts. Si les récentes études supposent que les virus pourraient avoir une forte influence sur le contenu génétique des communautés microbiennes par des transferts horizontaux de gènes et la lysogénie, le contrôle de la structuration de la diversité microbienne et ainsi jouer un rôle fondamental dans la régulation des cycles biogéochimiques, peu d'hypothèses sont émises concernant les eucaryotes.

Très peu d'études ciblant les communautés eucaryotes ont été entreprises. Cependant, les analyses basées sur l'ARNr et l'ARNm ont révélé l'activité des champignons dans les sédiments marins de subsurface (Edgcomb *et al*., 2011; Orsi *et al*., 2013a, 2013b). De façon intéressante, la détection de transcrits fongiques, au niveau de la marge du Pérou ainsi qu'au niveau du bassin de Canterbury, impliqués dans le métabolisme des glucides, des acides aminés et des lipides, suggère que les champignons sont acteurs du recyclage du carbone organique dans les sédiments marins (Orsi et *al*., 2013b). Codants essentiellement des hydrolases, les transcrits retrouvés dans ces deux études indiquent que les champignons sont capables de dégrader toute une variété de substrats organiques dans les sédiments marins profonds.

Les *Alphaproteobacteria* et *Betaproteobacteria* sont des acteurs majeurs dans la réduction des nitrates au sein de la subsurface (Orsi *et al*., 2013b). Dans cette même étude, il a été proposé que les nitrites issus de la réduction des nitrates pourraient être réduits par les *Gammaproteobacteria*, les *Firmicutes* mais aussi par les champignons. En effet, nos résultats soutiennent cette hypothèse car des transcrits fongiques impliqués dans la réduction des

nitrites ont détectés dans notre étude. Les communautés microbiennes de subsurfaces semblent donc agir de façon séquentielle dans le cycle de l'azote au sein des matrices sédimentaires.

L'expression fongique de sidérophores détectée au cours de cette thèse corrobore les résultats d'une précédente étude. En effet, plusieurs des levures isolées par Connell *et al*. (2009) au niveau du volcan sous-marin Vailulu'u, produisent des sidérophores, une molécule utilisée pour assimiler et d'utiliser le Fe(III) à partir de l'environnement et un isolat a été capable d'oxyder le Mn(II). Ces résultats indiquent que les communautés fongiques peuvent être impliquées dans le cycle biogéochimique du fer et du manganèse.

Les champignons seraient donc des acteurs non négligeables des grands cycles biogéochimiques comme celui du carbone, de l'azote et potentiellement de la séquestration de différents métaux dans les sédiments marins profonds.

3.4. Perspectives

Ce travail de thèse représente une première contribution et ouvre un nouveau chantier pour lequel de nombreuses perspectives se dégagent :

La diversification des conditions de culture

L'approche culturale est indispensable pour déterminer les potentiels physiologiques des différentes espèces présentes au sein de la biosphère profonde. Cependant, l'efficacité de culture des communautés microbiennes est estimée entre 0,001% et 1% (Amman *et al*, 1995). La mise au point de nouvelles techniques de culture à haut-débit innovantes devrait permettre de cultiver un grand nombre de micro-organismes incultivés à ce jour et ainsi d'étoffer la collection de culture déjà établie. De nombreuses approches à haut-débit ont été mises au point ces dernières années. La micro-encapsulation permet par exemple l'isolement de micro-colonies dans des micro-billes d'agarose. Les billes sont incubées sous un flux constant de milieu appauvri en nutriments et ensuite triées par cytométrie en flux. Cette approche qui conserve les échanges de métabolites et de signaux entre les cellules a été utilisée par Zengler

et al., (2005) et a permis de fournir plus de 10 000 isolats bactériens et fongiques à partir d'échantillons environnementaux. La technique d'extinction en milieu liquide pauvre en nutriments décrite par Stingl *et al.*, (2007) est une autre technique de culture à haut débit qui permet notamment la croissance de micro-organismes à croissance lente. Cette technique a permis l'isolement des premiers représentants des clades bactériens SAR11 (Rappé *et al.*, 2002) et OM43 (Connon et Giovannoni, 2002). A l'avenir, il serait souhaitable de multiplier les conditions de culture et en particulier en absence d'oxygène. En effet l'anaérobiose est un facteur qui n'a pas été testé lors de notre étude et bien que l'oxygène n'ait pas été mesuré lors de l'échantillonnage, il est fort probable qu'en profondeur l'environnement soit réduit. De nouvelles approches culturales sont aujourd'hui à l'essai afin d'améliorer l'efficacité de culture et de croissance des micro-organismes marins dans les habitats extrêmes comme par exemple la co-culture (Projet Européen MaCuMBA : Marine Microorganisms : Cultivation Methods for Improving their Biotechnological Applications). En effet, la communication entre cellules est un facteur important à prendre en compte car les cellules utilisent des molécules de signalisation pour coordonner leurs actions, et ces molécules joueraient un rôle important dans la stimulation de la croissance microbienne (Alain et Querellou, 2009). Pour comprendre les interactions entre les communautés fongiques et les conditions environnementales *in situ*, des cultures en bioréacteurs pourraient être envisagées afin de contrôler des paramètres importants tels que la pression, le pH ou la température (Zhang *et al.*, 2011).

Les capacités d'adaptation

Les capacités physiologiques et métaboliques des champignons marins de la biosphère profonde restent mal connues. Le séquençage de génome couplé à des approches de transcriptomique et de protéomique de certains représentants fongiques de la collection isolés à différentes profondeurs devra être envisagé afin d'identifier des gènes potentiellement impliqués dans les capacités d'adaptation. Ces données pourraient nous apporter des informations cruciales sur la dynamique fonctionnelle des communautés fongiques de la biosphère souterraine.

L'approche fonctionnelle

Les récentes études consacrées à la biosphère profonde ont apporté de nombreuses informations concernant la distribution et l'importance de la biosphère marine profonde mais de nombreuses questions restent encore sans réponses sur le rôle de ces organismes *in situ*. Bien que des premiers indices de division cellulaire, parmis les trois domaines du vivant, aient été mis en évidence dans des sédiments profondément enfouis, nous ne savons pas à quel taux de renouvellement se reproduisent les communautés de subsurface ou bien même si certains micro-organismes vivent sans se diviser durant des millions d'années (Colwell et D'Hondt, 2013). Il est à présent crucial de déterminer l'impact des communautés de subsurface sur les cycles biogéochimiques et de comprendre comment les micro-organismes persistent dans ces environnements extrêmes. L'approche fonctionnelle devrait nous éclairer sur les mécanismes mis en place par les communautés de subsurfaces pour survivre pendant de très longues périodes dans la lithosphère océanique.

Les interactions entre communautés

Les interactions entre communautés dans les sédiments marins sont encore mal connues et de nombreuses questions sur le fonctionnement global de cet écosystème restent en suspens. Bien que peu de données soient disponibles sur les virus associés à la biosphère profonde, ces derniers pourraient jouer un rôle fondamental dans la régulation des cycles biogéochimiques, la structuration de la diversité microbienne, et influencer le contenu génétique des communautés hôte par des transferts horizontaux de gènes et la lysogénie. En effet, la présence de nombreux virus lysogènes associés à des isolats bactériens a été démontrée au sein de la biosphère profonde (Engelhardt *et al.*, 2011). A notre connaissance, les virus associés aux champignons de subsurface n'ont pas été étudiés. Puisque les mycovirus sont largement répandus parmi les grands groupes taxonomiques de champignons (Liu *et al*., 2014), il serait intéressant de vérifier si des séquences virales sont présentes dans les génomes fongiques des sédiments marins profonds.

L'analyse biogéographique

Lors de cette thèse, une seule carotte de sédiments très profonds, jusqu'à 1922 mbsf, collectée au niveau de la marge continentale du bassin de Canterbury (Nouvelle-Zélande), a été étudiée en tant que modèle d'étude. Nous avons ainsi décrit la distribution verticale des communautés fongiques dans les sédiments marins profonds en utilisant cette carotte de sédiment comme modèle d'étude. Dans l'objectif d'obtenir une vision globale de la diversité micro-eucaryote et fongique, d'autres analyses sont en cours au niveau de différentes régions du globe (mer Noire, mer Méditerranée, mer du Nord, Manche). Les résultats devraient nous éclairer sur la distribution de ces communautés à l'échelle globale dans les écosystèmes sédimentaires marins.

Le potentiel biotechnologique

D'un point de vue biotechnologique, les champignons filamenteux sont les eucaryotes les plus largement utilisés dans le secteur industriel (Damare *et al*., 2012). Leur capacité de production de métabolites secondaires tels que les antibiotiques a induit leur exploitation biotechnologique. Si les champignons terrestres ont montré leur importance de ce point de vue, les champignons marins constituent une ressource biologique peu explorée et donc largement inexploitée. L'étude de Rateb et Ebel en 2011 a fourni la preuve directe que les champignons marins pélagiques représentaient un groupe écologique capable de produire des composés uniques. Dans ce contexte, la collection de culture établie lors de cette thèse offre un potentiel biotechnologique à explorer. L'étude préliminaire que nous avons menée révèle la diversité génétique de la collection et un potentiel génétique pour la synthèse de métabolites secondaires ayant des activités biologiques potentiellement intéressantes (Chapitre 3). Ces résultats prometteurs devront être complétés par d'autres études consacrées spécifiquement à l'expression de ces gènes d'intérêt ainsi qu'à la mise en évidence et à l'étude des molécules produites.

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Annexes : Valorisation de la recherche

1. Publications scientifiques

a. Publications issues des travaux de thèse

Ciobanu M.C., Burgaud G., Dufresne A., Breuker A., **Rédou V.**, BenMaamar S., Gaboyer F., Vandenabeele-Trambouze O., Lipp J.S., Schippers A., Vandenkoornhuyse P., Barbier B., Jebbar M., Godfroy A., Alain K. 2014. Microorganisms persist at record depths in the subseafloor of the Canterbury Basin. The ISME Journal. 8:1370–1380.

Mahé S., **Rédou V.**, Calvez T.L., Vandenkoornhuyse P., Burgaud G. 2014. Fungi in Deep-Sea Environments and Metagenomics. In: The Ecological Genomics of Fungi. Martin F. eds. pp. 325–354. Hoboken, NJ, USA.

Rédou V., Ciobanu M.C., Pachiadaki M.G., Edgcomb V., Alain K., Barbier G., Burgaud G.. 2014. In-depth analyses of deep subsurface sediments using 454-pyrosequencing reveals a reservoir of buried fungal communities. FEMS Microbiology Ecology. DOI: 10.1111/1574-6941.12447.

b. Contribution à d'autres publications

Burgaud G., Woehlke S., **Rédou V.**, Orsi W., Beaudoin D., Barbier G., Biddle J., Edgcomb V. 2013. Deciphering presence and activity of fungal communities in marine sediments using a model estuarine system. Aquatic Microbial Ecology. 70:45–62.

Dekov V.M., Bindi L., Burgaud G., Petersen S., Asael D., **Rédou V.**, Fouquet Y., Pracejus B. 2013. Inorganic and biogenic As-sulfide precipitation at seafloor hydrothermal fields. Marine Geology. 342:28–38.

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Deciphering the presence and activity of fungal communities in marine sediments using a model estuarine system

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ABSTRACT: Fungi are known to play key roles in ecologically important biogeochemical cycles and food webs. Most knowledge of environmental groups of fungi comes from terrestrial environments, and little is known about the potential for terrestrial fungi to colonize marine environments. We investigated the Delaware River estuary and bay as a model estuarine system to study the fungal community changes occurring along a transect from terrestrially influenced waters and sediments to a higher salinity, truly marine system. DNA-based clone libraries and a culture collection built using subseafloor sediment samples revealed that *Ascomycota* dominated the detected diversity ahead of *Basidiomycota* and *Chytridiomycota*. A clear transition in fungal communities from terrestrially influenced and low salinity environments to marine environments was visualized. A complementary RNA-based analysis coupled with fluorescence *in situ* hybridization of sediments indicated that only few fungi were metabolically active in marine sediments. Cultivation of pelagic and sedimentary fungi allowed clear identification and physiology testing of fungal communities of the Delaware Bay. Most isolates were affiliated to *Ascomycota* and *Basidiomycota*, and their growth was analyzed under different concentrations of salinity to test for habitat preference and degree of adaptability. Interestingly, most of the fungi isolated were halotolerant. The present study informs us on the source and fate of fungi that may be buried in the deep marine subsurface and is informative for future investigations of this environment.

KEY WORDS: Fungi · Subsurface sediments · Delaware Bay · DNA · cDNA · Cultivation

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INTRODUCTION

Fungi are known to play key roles in ecologically important symbioses, to represent a significant portion of the biomass in terrestrial systems and to play important roles in biogeochemical cycles and food webs (Bass et al. 2007, Gadd 2007). Despite the high number (~100 000) of fungal species described to date, this number may represent only ~13 % of fungal species on Earth (Hawksworth 2009). Recent studies have allowed increases in the understanding of deep marine environments that represent poorly described ecological niches for fungal communities (Edgcomb et al. 2002, López-García et al. 2003, 2007, Bass et al. 2007, Alexander et al. 2009, Burgaud et al. 2009, 2010, Edgcomb et al. 2011). The biogeochemical activities, composition and temporal and spatial dynamics of marine subsurface communities are an emerging topic in marine sciences and biogeochemistry, especially with the recognition that subsurface microbial communities may rival terrestrial ecosystems, both in terms of diversity or complexity. Cover-

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ing more than two-thirds of the Earth's surface, marine sediments could include between 5 and 15% of the Earth's active microbial biomass (Kallmeyer et al. 2009) and may represent a major reservoir of life, although this estimate was recently reduced (Kallmeyer et al. 2012). While some studies have focused on marine fungi in sediments (Raghukumar et al. 2004, Biddle et al. 2005, 2008, Damare et al. 2006, Bass et al. 2007, Lai et al. 2007, Nagano et al. 2010, Singh et al. 2010, 2011, Edgcomb et al. 2011), subsurface eukaryotic communities still remained largely underexplored. Initial investigations of subseafloor communities revealed low fungal diversity, yet fungi have been identified as likely dominant among eukaryotic microbial populations in deep-sea sediment cores of the Peru Margin and the Peru Trench (Edgcomb et al. 2011). Based on 1 to 35 m below seafloor (mbsf) sediment samples, Edgcomb et al. (2011) revealed a dominance of fungal sequences affiliated to *Basidiomycota* in clone libraries constructed from DNA and cDNA. The recovery of ribosomal RNA at these depths suggests that the microorganisms revealed were living cells rather than inactive spores or dead mycelia (Edgcomb et al. 2011), which may have global implications for ocean carbon and nitrogen cycling, given the extent of the deep subsurface biosphere (Oger & Jebbar 2010). Some fungal sequences retrieved from the Peru Margin represented potentially novel taxa, supporting the fact that marine sediments harbor a large fraction of undescribed fungi (Nagahama et al. 1999, 2003, 2006, 2008). Fungal diversity retrieved by culture-dependent methods in the deep-sea (Mouton et al. 2012, Singh et al. 2010, Edgcomb et al. 2011) also revealed that many of the fungal sequences detected were phylogenetically related to terrestrial fungi, raising ecological questions regarding the abilities of terrestrial fungi to adapt to deep subsurface conditions (low temperatures, high hydrostatic and lithostatic pressures and relative oligotrophy).

The boundaries between terrestrial and marine fungi are currently not clear, with the most widely accepted definition of a marine fungus as being those that grow and sporulate exclusively in a marine or estuarine habitat (Kohlmeyer & Kohlmeyer 1979). Since fungi are increasingly found in deep-sea environments using both culture-dependent and culture-independent methods, a clear differentiation between marine and terrestrial fungi has been lost (Edgcomb & Biddle 2011). There is some evidence that terrestrial/surface-dwelling fungi may be capable of colonizing deep-sea habitats due to their ability to alter their membrane composition to accommodate high hydrostatic pressure (Fernandes et al. 2004). The examination of marine fungi at this point is in need of a detailed biodiversity study to determine, using molecular, microscopic and cultivation approaches, what constitutes a marine fungus and whether there are members of the fungi that are active in the subsurface or if those organisms are merely preserved (either dead or inactive). As future microbiology expeditions targeting marine subsurface sediments and the deep biosphere are planned, it is important to be able to accurately interpret the potential significance of finding particular fungal groups in diverse marine subsurface samples. Additionally, the potential adaptation to, and survival of, terrestrial fungi in the marine subsurface needs to be explored. We chose the Delaware River estuary as a model estuarine system to study the fungal community changes occurring along a transect from terrestrially influenced water and sediments to a higher salinity, truly marine system. Our aim was to determine, using molecular, microscopic and cultivation-based techniques, what portion of detected fungi represents truly marine forms vs. inactive or active and highly adaptable, opportunistic terrestrial fungi and to gain insights into their levels of activity.

MATERIALS AND METHODS

Site description

The Delaware Bay was chosen as a model estuarine system since 3 major ecological zones could be distinguished easily: (1) an upper zone (Stn 23) characterized by fresh water under tidal influence, (2) a transition zone (Stn 11) with a wide range of salinity, high turbidity and low primary biological productivity and (3) a lower bay zone (Stn 1), extending to the Atlantic Ocean, with higher salinity and the highest levels of primary biological productivity (Santoro 2004) (Fig. 1).

Water and sediment sampling

Samples were collected using the RV 'Hugh Sharp' (Table 1). Water samples were collected using a submersible pump 1 m below the surface at 3 different stations representing the 3 major salinity zones of the Delaware Bay (Table 1).

Water samples were used for cultivation-based approaches and *in situ* hybridization. Regarding cultivation, water samples were stored at 4°C for a few

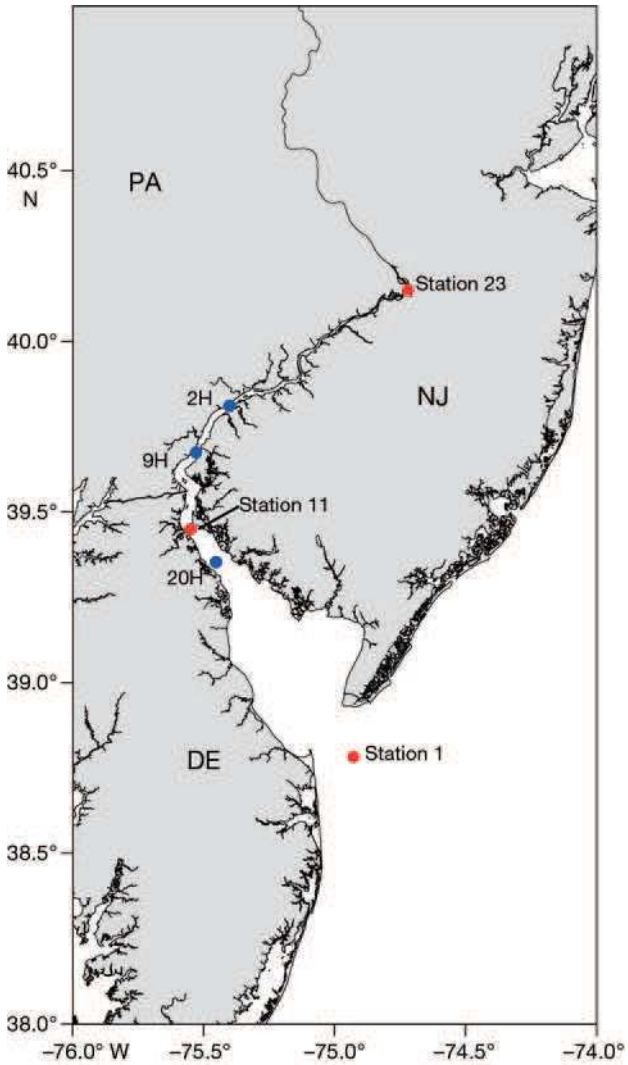


Fig. 1. Overview of sample collections with positions of the 3 water-sampling stations (1, 11 and 23) and the 3 sediment-sampling sites (2H, 9H and 20H). PA: Pennsylvania; NJ: New Jersey; DE: Delaware

hours before filtration and plating. For *in situ* hybridization, 200 ml of water samples were directly fixed with paraformaldehyde (2% final concentration) for 2 h at 4°C in the dark before filtration on polycarbonate filters (0.8 µm, Millipore), with a pre-filtration through 200 µm mesh (Pernthaler et al. 2001).

Bottom sediment samples were obtained on the same cruises using a KC Denmark HAPS corer, which recovers undisturbed samples of the sediment-water interface. The cores were extruded vertically aboard the ship and sectioned in 2 cm intervals. The sediment was placed in Whirl-pak sterile bags and frozen at −80°C. Samples were processed from the 2 to 4 cm below seafloor (cmbsf) depth horizon to avoid seawater contamination.

Sediment samples were used for cultivation-based approaches, *in situ* hybridization and calcofluor staining as well as analyses of small subunit ribosomal RNA (SSU rRNA) and internal transcribed spacer region 1 (ITS1) sequences. For *in situ* hybridization, multiple aliquots of 100 mg sediment samples were fixed in 2% paraformaldehyde (final concentration) for at least 2 h at 4°C in the dark. After fixation, sediment samples were washed 2 times with sterile 1× PBS and stored at −20°C in 50:50 1× PBS:96% ethanol (Pernthaler et al. 2001). Multiple 2 g aliquots were directly frozen at −80°C for extraction of nucleic acids. A summary of samples collected and methods applied to each sample can be found in Table 1.

Fungal isolation from sediment and water samples and salinity tolerance assays

Cultures were prepared aerobically at 25°C and atmospheric pressure using GYPS0, GYPS3, MEA0

Table 1. Delaware estuary sample collection metadata

Station	Sample	Date sampled	Latitude (N)	Longitude (W)	Salinity (PSU)	Study performed
23	Water	11 Mar 10	40° 08.96′	74° 43.31′	0.1	Microscopy
	Water	4 Jun 11			0.08	Culturing, salinity assay
11	Water	11 Mar 10	39° 26.916′	75° 33.125′	6.9	Microscopy
	Water	3 Jun 11			2.58	Culturing, salinity assay
1	Water	10 Mar 10	38° 46.94′	74° 55.76′	31.5	Microscopy
	Water	3 Jun 11			29.23	Culturing, salinity assay
2H	Sediment	12 Mar 10	39° 48.714′	75° 24.167′	0.19	Culturing, microscopy, DNA/cDNA, environmental cloning
9H	Sediment	12 Mar 10	39° 40.442′	75° 31.774′	1.5	Culturing, microscopy, DNA/cDNA, environmental cloning
20H	Sediment	11 Mar 10	39° 21.143′	75° 27.175′	9.2	Culturing, microscopy, DNA/cDNA, environmental cloning

and MEA3 culture media and a laminar flow hood. The GYPS medium contained the following components per liter: glucose 1 g, yeast extract 1 g, peptone 1 g, starch 1 g and agar 30 g. Sea salts (3%) were added to GYPS3. The MEA medium contained the following components per liter: maltose 12.75 g, dextrin 2.75 g, glycerol 2.35 g, peptone 0.78 g and agar 30 g. Sea salts (3%) were added to MEA3. All the culture media were supplemented with 500 mg chloramphenicol and 250 mg vancomycin per liter. For water samples, 20 ml of the 3 different suspensions were filtered on sterile 0.45 µm cellulose acetate filters and then deposited on the culture media. For sediment samples, 3 suspensions were processed (no dilution, 1:10 and 1:100) with sterile water amended with different sea salt concentrations depending on the salinity of the site sampled. Aliquots of 0.2 ml of each suspension were plated on each culture medium and spread with sterile flat rakes. Duplicates were processed for each condition. Pure cultures were obtained by streaking yeasts and central picking filamentous fungi on their respective enrichment media, and these pure cultures were kept in our culture collection for physiological analysis, morphological characterization and molecular identification. Nutrient plates exposed within a laminar flow hood during our isolation procedure were processed as a control. No fungal colonies appeared on our control plates, indicating no occurrence of aerial contaminations during isolation.

The filamentous fungal strains isolated were grown on solid GYPS medium. The effect of salinity on growth was assessed by modifying sea salt concentrations in culture media (0, 1.5 and 3%). Colony diameters were measured each 2 d for a maximum of 10 d to characterize fungal growth. All experiments were processed in triplicate. Regarding yeasts, the same procedure was established except that growth rate was visually estimated.

Nucleic acid extraction and PCR from sediment samples and fungal isolates

Sediment cores

RNA extractions were processed on frozen sediment cores (−80°C) with a sterile metal spatula. To avoid any seawater contamination, scrapings were taken from the central part of each different core (House et al. 2003). RNA was isolated from 2 g samples for each sediment core (4 RNA extractions of 0.5 g sediment) using the Fast RNA Pro Soil-Direct kit (MP Biomedicals) according to the manufacturer's instructions with few modifications, i.e. a treatment with 0.02 U Turbo DNAse with 1× Turbo DNAse Buffer (Invitrogen) and 15 mM EDTA for 30 min at 37°C followed by 10 min denaturation at 75°C. The Super Script III One-Step RT-PCR System (Invitrogen) was then used to reverse-transcribe total RNA to cDNA (following the manufacturer's instructions), and this step incorporated the first PCR amplification with f-ITS1F/r-ITS4 (for ITS locus amplification) or with f-EF4/r-EF3 (for SSU rRNA gene amplification). As fungal biomass was low, we processed a second nested PCR reaction. The GoTaq kit was used with f-ITS1F/r-ITS2 or f-EF4/r-Fung5. RNA samples were tested for DNA contamination by PCR amplification prior to reverse-transcription to control for complete elimination of residual DNA. All sediment-core RNA samples passed this test (no PCR amplification was detectable after 40 PCR cycles) and were used to prepare cDNA libraries. RNA yield was quantified using a Qubit fluorometer (Life Technologies), and cDNA was quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). ITS rRNA gene sequences were obtained using primers designed by White et al. (1990): ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′- TCC TCC GCT TAT TGA TAT GC-3′), followed by a second nested PCR reaction with ITS1F and ITS2 (5′- GCT GCG TTC TTC ATC GAT GC-3′). For 18S rRNA gene sequences, we used a primer set described by Smit et al. (1999), EF3 (5′-TCC TCT AAA TGA CCA GTT TG-3′) and EF4 (5′-GGA AGG GNT GTA TTT ATT AGA T-3′), followed by a second nested PCR reaction with EF4 and Fung5 (5′-GTA AAA GTC CTG GTT CCC C-3′). All PCR reactions were performed in 25 µl reaction volumes containing 24 µl of 1× PCR buffer (Promega), 2 mM of $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.6 mM of primers (forward and reverse), 1.25 U of GoTaq Polymerase (Promega) and 1 µl of DNA. The cycling parameters were as follows: after the cDNA synthesis step (55°C for 30 min), 94°C for 2 min, followed by 39 cycles of 95°C for 20 s, 55°C for 30 s and 68°C for 40 s, and a final 5 min at 68°C. Regarding the second nested PCR reaction, we used the following conditions: 94°C for 5 min, followed by 30 cycles of 95°C for 40 s, 55°C for 40 s and 72°C for 2 min and a final 7 min at 72°C. PCR amplifications from DNA prepared from sediment samples were conducted using the same procedure but only on ITS rRNA gene sequences.

Fungal isolates

DNA of fungal isolates was extracted with a Fast DNA Spin Kit (MP Biomedicals) according to the manufacturer's instructions. For filamentous fungi, the 18S rRNA gene and ITS were amplified and sequenced. SSU rRNA gene sequences were amplified with NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′), NS3 (5′-GCA AGT CTG GTG CCA GCA GCC-3′) and ITS5R (5′-CCT TGT TAC GAC TTT TAC TTC C-3′) primers (White et al. 1990). ITS were amplified using ITS1F and ITS4 primers. For yeasts, amplifications of the D1/D2 region of the 26S rRNA gene were carried out with rRNA gene primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG-3′), LR6 (5′-CGC CAG TTC TGC TTA CC-3′), NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) as described by Gadanho & Sampaio (2005). All PCR reactions were performed in 20 µl reaction volumes containing 1× GoTaq Buffer (Promega), 2 mM of each of the 4 dNTPs (Promega), 0.08 mM of each primer (Proligo), 1 U of GoTaq DNA polymerase (Promega) and 1 µl of genomic DNA. PCR reactions used a PTC-200 thermal cycler (MJ Research). The PCR temperature profile consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C and 2 min at 72°C and a final extension step of 2 min at 72°C before a conservation at 4°C. A negative control in which DNA was replaced by sterile water was included. PCR products were evaluated by electrophoresis in 0.8% (w/v) agarose gel (Promega) in 0.5× Tris-borate EDTA (TBE) buffer at 90 V for 90 min and stained with ethidium bromide. The molecular size markers were Lambda DNA/EcoR1 + HindIII (Promega). DNA banding patterns were visualized under UV transillumination, and picture files were generated using Gel-Doc 2000 (Bio-Rad).

Environmental cloning of sediment samples and sequencing of fungal isolates

Sediment cores

The amplified SSU and ITS rRNA gene fragments obtained from cDNA prepared from sediment samples were cloned into the TOPO TA cloning vector pCR2.1 and transformed into TOP10 chemically competent *Escherichia coli* according to the manufacturer's instructions (Invitrogen). Transformants were selected by blue-white selection on Luria-Bertani agar plates containing ampicillin (100 µg ml^{-1}). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F and M13R. Clones were then sequenced by Genewiz.

Fungal isolates

Sequences obtained by Big Dye Terminator technology (Applied Biosystems) at the 'Ouest Génopole' sequencing facility in the 'Station Biologique de Roscoff' were edited and processed for quality control using SEQUENCHER v. 4.8 (Gene Codes). Sequences were then imported to MEGA 4.0 software (Tamura et al. 2007). Each sequence was analyzed to find GenBank sequences with closest BLASTn hits (Altschul et al. 1990). Similarities between sequences were assessed using pairwise distance calculation with MEGA 4.0 for the purpose of taxonomic assignment and calculating OTUs.

Phylogenetic analyses

Sequences were trimmed to ensure that all had the same start and end point and checked for chimeras using Bellerophon Chimera Check and the Check_Chimera utilities (Ribosomal Database Project) (Cole et al. 2003). All SSU and ITS rRNA gene sequences were aligned using CLUSTALW v. 1.83 (Thompson et al. 1994). After visual inspection and manual correction, the alignments were analyzed using MODELTEST v. 3.7 (Posada & Crandall 1998) to obtain the optimal evolutionary model for subsequent phylogenetic analyses.

The phylogeny of sediment SSU rRNA sequences obtained from cDNA was evaluated using maximum likelihood. For this approach, we used RAxML version 7.0 as implemented on the CIPRES Portal (www.phylo.org). The tree was constructed using an alignment of 503 unambiguous positions under the General Time Reversible (GTR)+I+Gamma model of sequence evolution. The tree contains the sequences of the most closely affiliated cultured representatives of each sequence based on BLASTn analyses against GenBank's nr database. The phylogenetic analysis included 47 sequences from Stn 2H, 56 from Stn 9H and 57 from Stn 20H. Bootstrapping and determination of the best estimate of the maximum likelihood (ML) tree topology for this data set was conducted with the Rapid Bootstrapping algorithm of RAxML. Rarefaction analysis was performed using the alpa_rarefaction.py command in QIIME (Caporaso et al. 2010).

Fluorescence *in situ* hybridization (FISH) and calcofluor staining for water and sediment samples

For sediment samples, 100 µl of fixed sediments were first treated to separate microbial cells from the sediment matrix as described by Kallmeyer et al. (2008). Briefly, cells were detached using a detergent mix (EDTA, Tween 80, sodium-pyrophosphate and methanol) and an ultrasonic treatment (Aquasonic model #75HT, 20 s), followed by density centrifugation through a cushion of Nycodenz (3000 × *g* for 10 min). Supernatants (~10 ml) were then filtered on 0.2 µm white polycarbonate filters (Millipore) using a gentle vacuum (<0.2 bar) before hybridization of the eukaryotic probe. For water samples, 20 ml of fixed water were filtered onto white polycarbonate filters using gentle vacuum (<0.2 bar).

Pieces of polycarbonate filters (3 per sediment and water sample) were cut in squares and pasted onto slides with one drop of 0.2% low-gelling point agarose (35 to 40°C) (Menzel-Glaser). All slides were then dipped in 0.2% agarose and air-dried. Samples were then subjected to dehydration with increasing concentrations of ethanol (50, 80 and 96%, for 3 min each). The concentration of working solutions of the eukaryotic probe Euk516-Cy3 (5′-ACC AGA CTT GCC CTC C-3′, Amann et al. 1995) was 30 ng l^{-1}. The hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.03% SDS, 20% formamide and the fluorescent probes were gently mixed in a ratio of 10:1 (v/v) to obtain a final oligonucleotide concentration of 3 ng l^{-1}. For hybridization, slides were placed in sampling tubes and incubated at 46°C in the dark for 3 h. Following hybridization, the slides were washed with pre-warmed washing buffer (20 mM Tris/HCl, 5 mM EDTA [pH 8.0] and 215 mM NaCl) for 20 min at 48°C. Slides were rinsed with double-distilled water, air dried, DAPI stained (final concentration 1 µg ml^{-1}) and mounted with the antifading reagent Citifluor AF 2 (Citifluor) before observation under a fluorescent microscope.

Calcofluor white staining was also used to examine fungal cells on pieces of the same polycarbonate filters described above. Slides were stained with an 0.5 mM solution of calcofluor white M2R (Sigma Aldrich) (4,4′-bis[4-anilino-6-bis{2-ethyl}amino-s-triazin-2-ylamino]-2,2′-disulfonic acid). The targets of calcofluor white M2R are chitin, cellulose and carboxylated polysaccharides. Following incubation in the dark for 5 min, the slides were washed with sterile water and observed using epifluorescence microscopy (with a Zeiss Axioplan 2 imaging microscope equipped with a Zeiss AxioCam camera).

Sequence accession numbers

Nucleotide sequences obtained in the present study were deposited in GenBank under accession numbers JX846650 to JX846808 (SSU rRNA sequences), JX967357 to JX967404 (ITS1 from DNA-based approach) and JX967405 to JX967502 (ITS1 from RNA-based approach). Sequences from cultured fungi (18S, ITS1 and 26S rRNA gene) were also deposited in GenBank under accession numbers JX967503 to JX967533.

RESULTS

Fungal isolation from sediment and water samples

A total of 141 fungal strains were isolated from the water of the different stations (Fig. 1, Table 1) using the 4 different culture media chosen in the present study. The GYPS base medium allowed us to cultivate 64 strains as well as the MEA base medium that produced 77 strains. The isolation ratio between salted and unsalted media gave different results depending on the isolation medium used; 28 strains were isolated from GYPS3 and 36 from GYPS0. For MEA, 51 strains were isolated from MEA3 and 26 strains from MEA0. More filamentous fungi were isolated from the Delaware Bay compared to unicellular yeasts: 84 filamentous fungi (60%) were retrieved compared to 57 yeasts (40%). The isolation ratio was dependent on the station sites since a pattern of distribution was observed. An increasing number of strains were retrieved as we transitioned from marine to more freshwater sites: 12 were obtained from Stn 1, 63 from Stn 11 and 66 from Stn 23, representing 8.5, 44.5 and 47% of all fungal strains isolated, respectively. Filamentous fungi and yeasts were mostly isolated from the almost freshwater Stn 23 and the brackish Stn 11. Only a few species were retrieved from the truly marine Stn 1, i.e. 7 yeasts and 5 filamentous fungi. We did not succeeded in isolating fungi from sediment samples.

Salinity tolerance assays

To visualize a pattern of distribution of filamentous fungi along a salinity gradient, diameters of growth of all strains after 3 d at 0 and 3% sea salt concentrations were plotted on a simple graph (Fig. 2A). A first clear clustering can be visualized between slow-growing strains (diameters <2 cm) and fast-growing strains

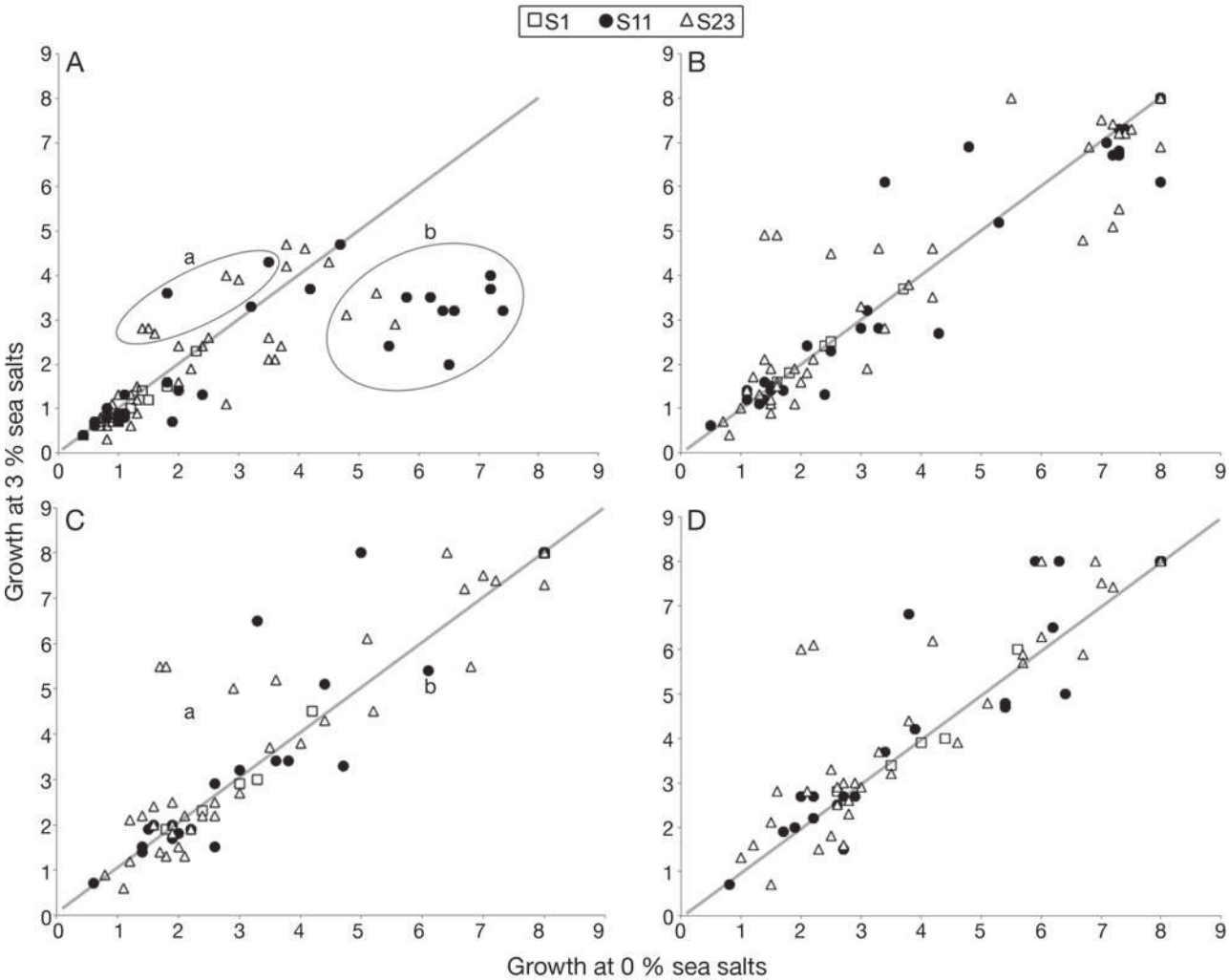


Fig. 2. Growth rate of filamentous fungal strains at 0 and 3% sea salts depending on the geographic origin (Stns 1, 11 and 23; see Fig. 1). Mycelial colony diameters (cm) were measured after (A) 3, (B) 5, (C) 7, and (D) 10 d

along a straight line that characterizes 58 halotolerant strains. We were able to extract a pattern of response for halophiles (7 strains in Cluster a) and non-halophiles (12 strains in Cluster b). Regarding halophiles, the same pattern was obtained after 5, 7 and 10 d of growth (Fig. 2B,C,D) although the growth difference between 0 and 3% sea salts was minimal. For non-halophiles, it appears that those strains were able to further adapt to higher salinity since after 5, 7 and 10 d of growth, Cluster b was not visible anymore, and the strains appeared halotolerant.

Regarding yeasts, only qualitative/semi-quantitative information was gathered depending on growth in culture media at different sea salt concentrations. All data were compiled in Table 2, and we were able to process a comprehensive analysis of the profile obtained and to form clusters with halophilic (9), slightly halophilic (4), halotolerant (21) and non-halophilic (23) yeast strains.

The isolation rate of yeasts depending on (1) sampling sites and (2) tolerance to sea salts indicated that, as shown for filamentous fungi, a pattern of distribution was clearly apparent and appears linked with salinity concentrations at the 3 different sites. Indeed, halophilic and slightly halophilic yeasts were mostly isolated from Stns 1 (marine) and 11 (brackish). More halotolerant yeasts were isolated from Stn 11 than Stn 23 (freshwater), and more non-halophiles were isolated from Stn 23 than Stn 11. Regarding filamentous fungi, non-halophiles were mostly isolated from Stns 11 and 23 (Fig. 2), but unexpectedly, the rare halophiles isolated were harvested more frequently from freshwater and brackish stations and not from the fully marine station.

Taxonomic assignments of fungal cultures were made on the basis of BLASTn analyses using the GenBank nr database (SSU, ITS and/or 26S rRNA genes) as detailed in Appendix 1, and assignments

Table 2. Physiological analysis of yeast strains. Growth was visually estimated on broth media at different sea salt concentrations (0, 1.5 and 3 %) and represented as 3 part bars

Yeast strain	Station	Salinity 3 - 1.5 - 0	Physiology
Y1	11		Non halophile
Y2	11		Halophile
Y3	11		Non halophile
Y4	11		Slightly halophile
Y5	11		Halotolerant
Y6	11		Halophile
Y7	11		Non halophile
Y8	11		Halotolerant
Y9	11		Halotolerant
Y10	23		Halotolerant
Y11	23		Halotolerant
Y12	1		Slightly halophile
Y13	11		Non halophile
Y14	11		Halophile
Y15	11		Non halophile
Y16	11		Halotolerant
Y17	23		Halotolerant
Y18	23		Non halophile
Y19	11		Non halophile
Y20	11		Halotolerant
Y21	1		Halophile
Y22	1		Non halophile
Y23	11		Halotolerant
Y24	11		Slightly halophile
Y25	23		Non halophile
Y26	23		Non halophile
Y27	23		Non halophile
Y28	23		Non halophile

Yeast strain	Station	Salinity 3 - 1.5 - 0	Physiology
Y29	1		Non halophile
Y30	1		Non halophile
Y31	11		Non halophile
Y32	11		Halotolerant
Y33	11		Halotolerant
Y34	11		Slightly halophile
Y35	11		Halophile
Y36	11		Halotolerant
Y37	11		Halotolerant
Y38	11		Non halophile
Y39	11		Halophile
Y40	23		Non halophile
Y41	23		Halotolerant
Y42	23		Halotolerant
Y43	23		Halotolerant
Y44	23		Non halophile
Y45	23		Halotolerant
Y46	11		Non halophile
Y47	11		Halophile
Y48	11		Halotolerant
Y49	11		Halophile
Y50	23		Non halophile
Y51	11		Non halophile
Y52	23		Non halophile
Y53	23		Halotolerant
Y54	1		Halotolerant
Y55	1		Non halophile
Y56	11		Halotolerant
Y57	23		Halophile

were confirmed by observations of morphology. Table 3 presents the identity of each cultured isolate and the salinity of the waters from which it was isolated. In most cases, we did not recover the same isolates from waters of different salinity. Exceptions were *Fusarium oxysporum, Leptosphaeria maculans* and *Cadophora luteo-olivacea*, which were isolated from both 0.01–0.008 % and 0.23–0.69 % salinity, and *Acremonium* sp., which was isolated from both 0.23–0.69 % and 2.93–3.15 % salinity.

Environmental cloning of sequences from sediment samples

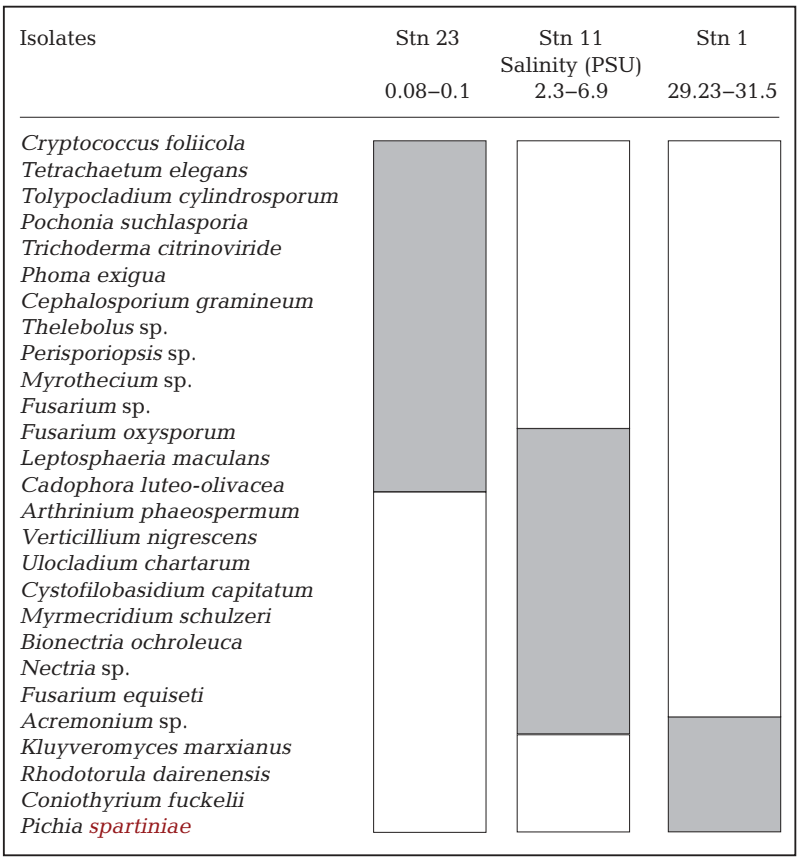
ITS from cDNA and DNA

We first utilized the ITS1 as a universal fungal barcode to reveal the fungal communities present in different sediment samples using DNA. Then, the active fraction of the fungal community was targeted by using cDNA that was reverse-transcribed from the total RNA extracted from each sediment sample.

For the DNA-based analysis, 192 cloned ITS1 sequences were analyzed for Stns 2H, 9H and 20H (equal sequencing effort for each station). After removal of 148 sequences that were either suspected chimeras, poor quality sequences or highly divergent sequences matching only 1 end of the SSU rRNA gene flanking the ITS1 sequence (with the remaining sequence not matching anything in GenBank), we only analyzed 10 clones from Stn 2H, 14 from Stn 9H and 24 from Stn 20H. These clustered into 8, 3 and 5 different taxonomic groups at 97 % sequence similarity, respectively (Table 4).

For the cDNA-based analysis of ITS1, 192 clones were also processed for all stations. After the removal of 83 poor-quality sequences (see above) and 11 sequences affiliating with choanoflagellates, we were able to analyze 2 clones from Stn 2H, 65 from Stn 9H and 31 for Stn 20H. Diversity obtained using the RNA-based analysis was clearly lower than for the DNA-based approach, as clones from Stns 2H, 9H and 20H clustered into only 3 OTUs at 97 % sequence similarity. Clones from 9H and 20H produced only 1 identical OTU (Table 5).

Table 3: Presence (grey) and absence (white) map of cultured fungal isolates and their isolation sites



SSU rRNA gene from cDNA

The active fraction of the fungal community in our sediment samples was also targeted by using cDNA prepared as above to amplify the eukaryotic SSU rRNA genes present in each sample. A total of 192 clones were sequenced for these analyses (equal sequencing effort for each of the 3 stations), producing 155 sequences after removal of poor-quality sequences or suspected chimeras (as above). A total of 57 sequences were retained for downstream analysis from Stn 20H, 56 from Stn 9H, and 47 from Stn 2H.

Phylogenetic analysis

A total of 59 non-redundant sequences obtained after OTU clustering recovered from analysis of SSU rRNA genes based on cDNA prepared from sediment samples (Fig. 3A) affiliated with members of the *Basidiomycota* and *Chytridiomycota*: 3 from Stn 2H (0.19 PSU salinity) and 55 from Stn 9H

Table 4. Sequence-based identification of OTUs (DNA-based analysis)

Station	Name / No. of clones	Close neighbor	%ID	Environments	Accession no.	Source
2H	OTU1 / 1	Uncultured fungus clone	93.9	Phyllosphere	FJ758448	Jumpponen & Jones (2009)
2H	OTU2 / 2	*Cochliobolus lunatus*	97.3	Plants	JN107740	Unpublished
2H	OTU3 / 1	*Cryptococcus aquaticus*	98.0	Lakes, cold ecosystems	AF410469	Scorzetti et al. (2002)
2H	OTU4 / 2	Uncultured fungus clone	98.4	Rhizosphere	AJ920023	Paradi & Baar (2007)
2H	OTU5 / 1	*Scutellospora heterogama*	81.0	Plant rhizosphere	FM876839	Krüger et al. (2009)
2H	OTU6 / 1	Uncultured fungus clone	83.3	Mangrove sediments, dead leaves	JQ038341	Fasanella et al. (2012)
2H	OTU7 / 1	*Rhizophydium* sp.	97.5	Soil	DQ485685	Letcher et al. (2006)
2H	OTU8 / 1	Uncultured fungus clone	100	Phyllosphere	JN905553	Unpublished
9H	OTU1 / 5	*Fibulochlamys* sp.	100	Soil	FM955450	Madrid et al. (2010)
9H	OTU2 / 7	Uncultured fungus clone	98.4	Deep-sea sediments	DQ279844	Lai et al. (2007)
9H	OTU3 / 2	Uncultured fungus clone	83.9	Rhizosphere	GU366680	Yarwood et al. (2010)
20H	OTU1 / 5	*Pestalotiopsis maculiformans*	98.7	Dead leaves, leaf litter	EU552147	Marincowitz et al. (2008)
20H	OTU2 / 4	*Lycoperdon pyriforme*	100	Decaying logs	AY854075	Unpublished
20H	OTU3 / 3	Uncultured *Chytridiomycota*	79.2	Lakes	HQ191408	Monchy et al. (2011)
20H	OTU4 / 5	Uncultured fungus clone	92.5	Mangrove sediments, dead leaves	JQ038342	Fasanella et al. (2012)
20H	OTU5 / 7	*Phaeosolania densa*	89.9	Decaying wood	AY571056	Bodensteiner et al. (2004)

Table 5. Sequence-based identification of OTUs (cDNA-based analysis)

Station	Name/ No. of clones	Close neighbor	%ID	bp/bp	Environments	Accession no.	Source
2H	OTU1 / 2	Uncultured fungus clone	96.5	246/255	Phyllosphere	JN904921	Unpublished
9H	OTU1 / 65	*Rhodotorula glutinis*	99.3	272/274	Deep-sea environments	AB025993	Nagahama et al. (2001)
20H	OTU1 / 31	*Rhodotorula glutinis*	99.3	272/274	Deep-sea environments	AB025993	Nagahama et al. (2001)

(1.5 PSU salinity). The nearest cultured relatives of these sequences were *Nematoloma* and *Cortinarius*. One sequence from Stn 2H was affiliated to *Chytridiomycota* (nearest relative an uncultured *Spizellomyces*).

The recovered sequences that affiliated with the *Ascomycota* were broadly distributed over several *Ascomycota* classes. Eight sequences from Stn 2H (0.19 PSU salinity) affiliated with relatives of *Preussia, Phoma, Westerdykella* and *Eremodothis*. Five sequences from Stn 2H affiliated with *Dothideomycete* sp., and 10 affiliated with members of *Penicillium*, *Thysanophora* and *Aspergillus*. A total of 57 sequences from Stn 20H (9.2 PSU salinity) and 1 sequence from Stn 9H (1.5 PSU salinity) also affiliated with *Aspergillus*. Ten sequences from Stn 2H affiliated with species of *Capronia* and *Phialophora* and with an uncultured fungus from soil. Four sequences from Stn 2H affiliated with species of *Trichoderma*, and 4 affiliated with *Phialophora*. However, diversity was only partially described since the accumulation curves did not reach saturation (Fig. 3B).

FISH and calcofluor staining for water and sediment samples

Calcofluor-stained fungal cells were visualized from water samples collected from Stns 11 and 23. No fungal cell was ever visualized from Stn 1. Multicellular conidia with a short conical beak were retrieved from Stn 11 (Fig. 4A). Fungal spores showing a blastic conidiogenesis are typical members of the *Ascomycota*. The conidium in Fig. 4A appears morphologically similar to *Alternaria*, an ubiquitous dematiaceous fungus. One tetraradiate conidium with long arms of equal length (40 µm) and showing phialidic conidiogenesis was visualized from water collected at Stn 23 (Fig. 4B) that is typical of *Lemonniera* sp. Stained fungal spores were also visualized from sediment samples of Stns 2H and 9H (Fig. 4C–F), although their presence was sporadic. No fungal cells were visualized from Stn 20H, despite multiple attempts.

FISH coupled with calcofluor staining also allowed us to detect active fungal cells in sediment samples from Stn 2H (0–2 and 2–4 cmbsf), representing the

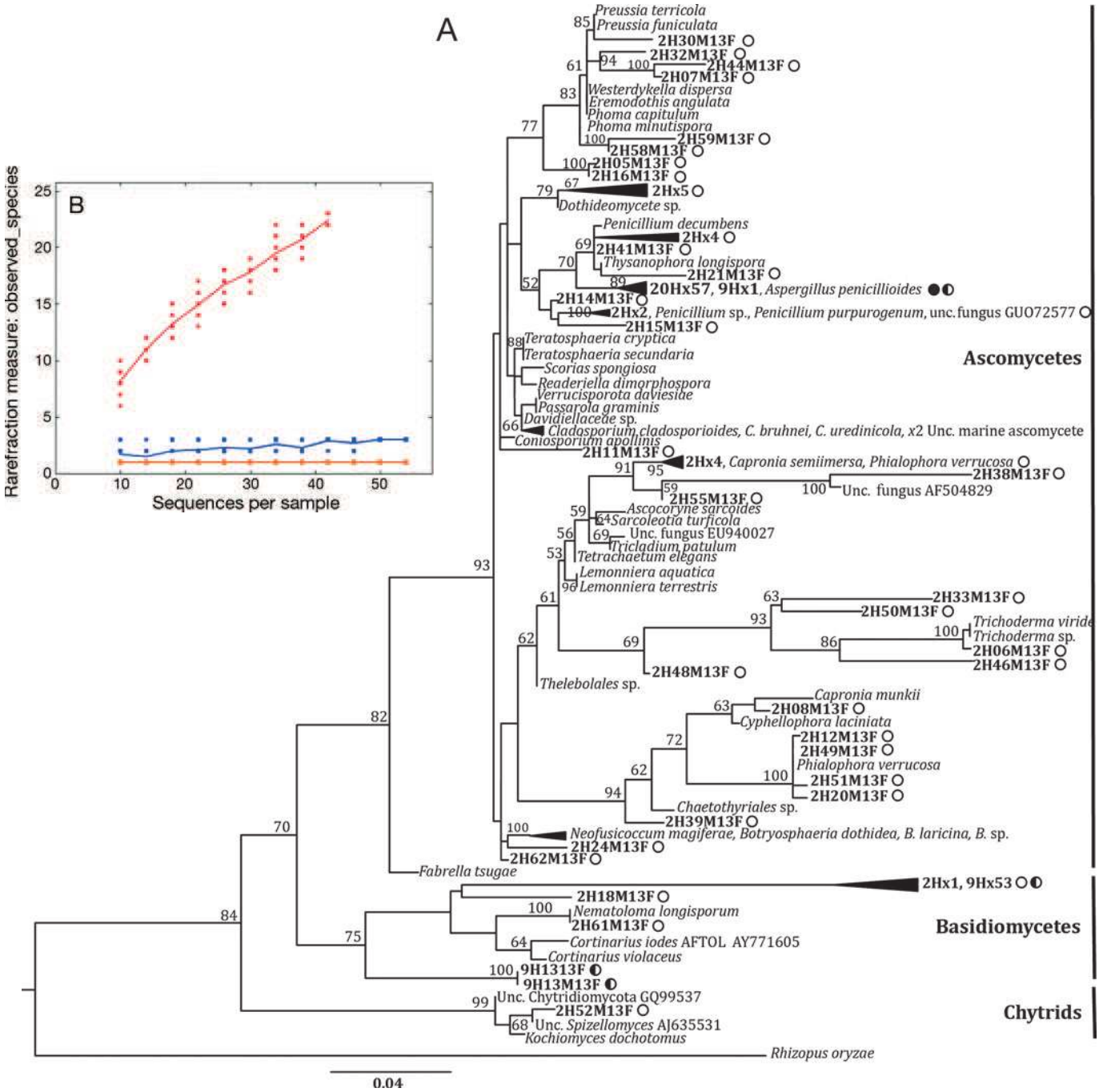


Fig. 3. SSU rRNA-based phylogeny (maximum likelihood) of sediment fungal sequences obtained from cDNA-based analyses. Numbers at nodes are bootstrap values ≥50 % under (A) maximum likelihood and (B) rarefaction curves. Environmental sequences are marked according to geographic origin: (white circles) Stn 2H, (black and white circles) Stn 9H and (black circles) Stn 20H. Unc.: uncultured

nearly freshwater station. Fungal cells appeared red/pink with blue cell walls since we used a Cy3 eukaryotic probe and calcofluor (Fig. 4D,G). Few fungal cells were observed in samples prepared from marine sites.

DISCUSSION

Our research objectives were to study the fungal community changes occurring along a transect from the freshwater zone to marine waters utilizing metho-

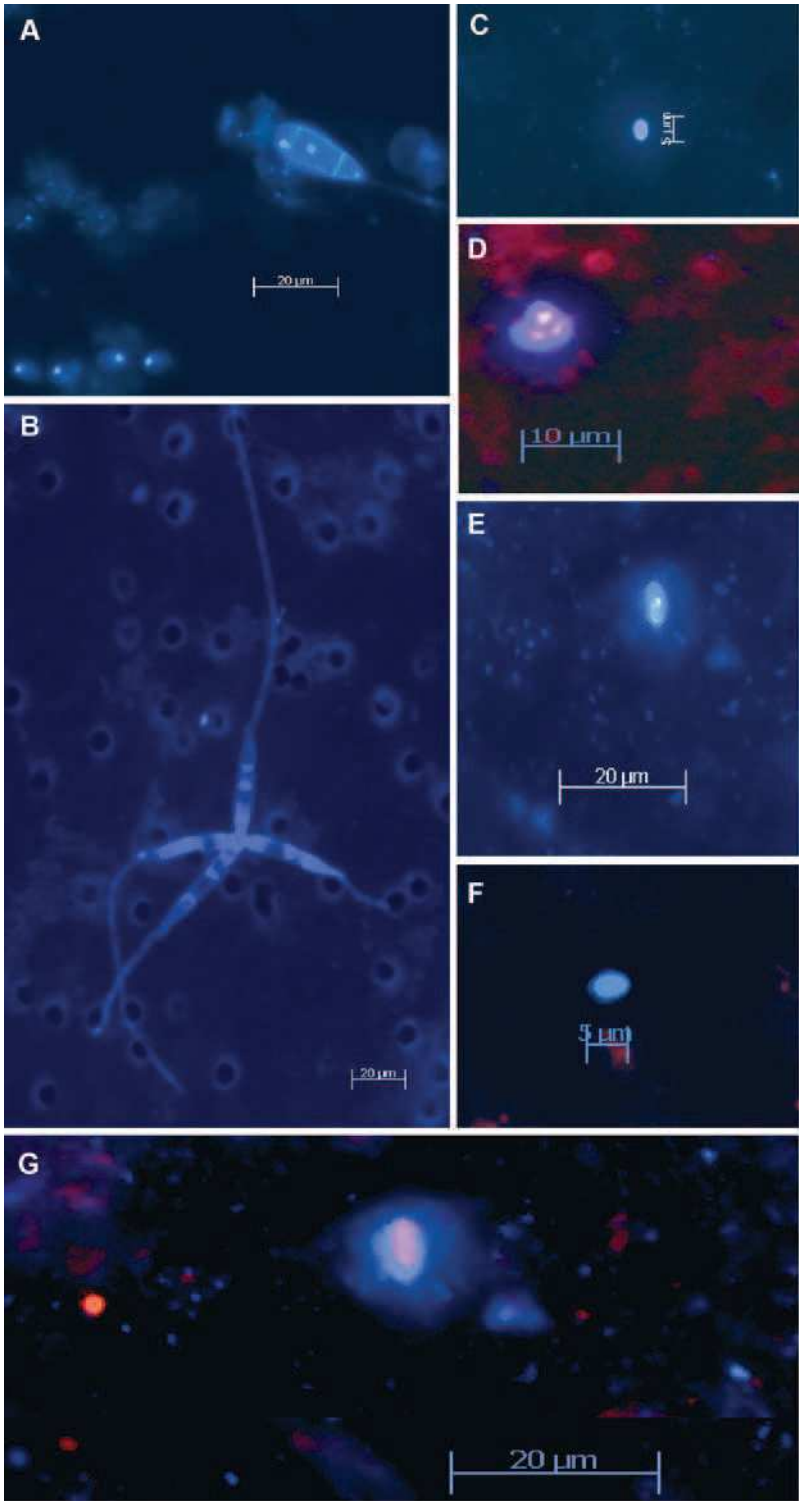


Fig. 4. Observation of fungal cells using calcofluor/DAPI staining and fluorescence *in situ* (FISH) from filtered waters and sediments. (A) Filtered water from Stn 11: 20 ml through 0.8 µm; stained with calcofluor and DAPI; bar = 20 µm. (B) Filtered water from Stn 23: 20 ml through 0.8 µm; calcofluor; bar = 20 µm. (C) Sediments from Stn 2H (0–2 cmbsf); calcofluor; bar = 5 µm. (D) Sediments from Stn 2H (0–2 cmbsf); calcofluor and FISH; bar = 10 µm. (E) Sediments from Stn 2H (2–4 cmbsf); calcofluor and DAPI; bar = 20 µm. (F) Sediments from Stn 9H (0–2 cmbsf); calcofluor; bar = 5 µm. (G) Sediments 2H (2–4 cmbsf); calcofluor and FISH; bar = 20 µm

dologies to segregate between the active and inactive fraction of fungi. The main aim was to better interpret the significance of fungal signatures being increasingly found in subsurface environments using both culture-dependent and culture-independent methods.

Evidence for a distribution pattern

Success in fungal isolation was dependent on the salinity of the different sites since a decreasing number of fungal strains, both yeasts and filamentous fungi, were harvested as we transitioned from freshwater to marine sites. Another correlation was observed when comparing isolation sources and physiology (Fig. 2, Table 2), showing that the yeasts from marine sites exhibited growth consistent with a strong preference for higher salinity, while the isolates from brackish sites were haloterant, and the isolates from fresh waters were clearly not halophiles. Indeed, based on the culturing results, each site/salinity condition harbored a mostly distinct fungal community (Table 3). Interpretation appeared more complex for filamentous fungi and showed that those organisms are more able to adapt to different salinities.

Few fungi were retrieved in culture at >1 site, despite using the same enrichment media. While it is possible that expanded culturing efforts using additional filtered water samples as inocula would have produced a greater number of overlapping cultures among sites, we think the observed pattern of culture recovery suggests very different active fungal populations in waters of significantly different salinity. This is particularly striking when one compares the successful isolates from Stn 23 (0.008 to 0.01 % salinity) to those from Stn 1 (2.92 to 3.15 %) (Table 3). If our cultures were being seeded primarily by inactive spores, it is likely that we would observe a greater overlap in our culturing results for the different stations.

Molecular results from SSU rRNA and ITS1 region analyses support the idea of a transition in fungal community structure along the salinity gradient since a much narrower spectrum of taxonomic signatures was recovered from the fully marine sediments.

The same pattern was also confirmed using FISH and calcofluor staining since most of the fungal cells visualized were from brackish and freshwater sediment samples (Fig. 5). The observed conidia indicated the presence of fungi, although the fungal biomass appeared insignificant. Abundant visible aggregations of organic particles in our water samples resulted in a strong underestimation of fungal biomass using microscopy-based approaches since many cells were trapped in these micro- and macro-aggregates. Moreover, many unstained dead/broken hyphae were also visualized in all sediment samples studied, which seems to indicate that some fungal populations settle from the water column into the sediments but have no ecological role in such environments. These results influenced our choice to work primarily with cDNA to reveal the molecular diversity of fungal communities in sediments since RNA-based approaches minimize the influence of dead cells.

Fungal abundance and diversity appeared to be inversely correlated with increasing salinity, as revealed recently in a study of saline, freshwater and brackish marshes, where fungal diversity and composition were influenced by both salinity, shaping the fungal composition, and the presence of plants, strengthening the fungal diversity (Mohamed & Martiny 2011). There are likely other factors that also influence fungal diversity and abundance, such as bacterioplankton (Grubisic et al. 2012) and eukaryotic photosynthetic producer communities (Gutiérrez et al. 2011) that are known to shape heterotrophic fungal communities in freshwater, brackish and marine sediment ecosystems.

Diversity of culturable fungi and tolerance to salinity

The decline in fungal diversity from more freshwater to fully marine samples is also reflected by the results of the culture-based studies. Isolations from the fresh waters at Stn 23 produced 14 different isolates, the brackish waters at Stn 11 produced 12, and the fully marine Stn 1 produced only 5 (Table 3). Three of the isolates overlapped between Stns 23 and 11 (*Fusarium oxysporum, Leptosphaeria maculans* and *Cadophora luteo-olivacea*), and only 1 isolate was recovered from water from Stns 11 and 1 (*Acremonium* sp.). Those overlapping isolates are congruent with their ecology since *F. oxysporum* and *L. maculans* are plant pathogens (Diener 2012, Pedras & Sarma-Mamillapalle 2012). *C. luteo-olivacea* has been described as a wood-destroying soft-rot fungus (Blanchette et al. 2004) and has also been isolated from lakes (Gonçalves et al. 2012). *Acremonium* represents a genus found in several marine environments, including deep-sea sediments (Singh et al. 2010) and seaweed (Zuccaro et al. 2008). Together with molecular results and the discussion above, this suggests that the active fungal community transitions in composition along this salinity gradient and that many fungi found in fresh waters are not adapted for survival in the fully marine environment. This is consistent with previous findings of a relatively simple fungal community composition in marine systems (based on fungal-specific clone library analyses), with relatively few phylotypes in total relative to terrestrial systems (Bass et al. 2007, Cathrine & Raghukumar 2009, Le Calvez et al. 2009, Edgcomb et al. 2011).

An increasing number of fungal isolates, filamentous fungi and yeasts were recovered from more fresh waters. Only 12 fungal strains were recovered from the fully marine Stn 1, while 63 and 66 strains were recovered from Stns 11 and 23, respectively. In agreement with Richards et al. (2012), this suggests that fewer fungi are adapted to life in fully marine waters and that a greater diversity exists in brackish and fresher waters. Our results support the notion of a gradient in fungal populations along this transect, with fungi that are adapted to marine conditions isolated from the fully marine site, non-halophiles isolated from the almost freshwater site, and some strains with modest abilities to tolerate saline conditions isolated from brackish conditions.

The discovery of halotolerant fungi of likely terrestrial origin is consistent with other studies of deep-sea and subsurface marine sediments where many signatures affiliated with clades of known terrestrial fungi (Edgcomb et al. 2011, Takishita et al. 2006). Some yeasts are known to be capable of altering their membrane composition to accommodate high hydrostatic pressure under short-term experimental conditions (Fernandes et al. 2004). Such an adaptation would be required of any terrestrial fungi dispersed to the marine environment and subsequently deposited in deep marine sediments. The chitin-rich cell walls of fungi likely confer an advantage when it comes to adapting to higher osmotic pressure and the stresses of osmotrophic feeding (reviewed by Richards et al. 2012).

Molecular diversity in sediments

The SSU rRNA analysis indicates 3 very different communities along this salinity gradient, but a community dominated in all cases by Dikarya (the vast majority of sequences affiliated with ascomycetes, followed by basidiomycetes) (Fig. 3). This is not surprising since the dominance of Dikarya has already been observed in deep oceans, deep-sea sediments and anoxic sediments (Bass et al. 2007, Edgcomb et al. 2011, Cathrine & Raghukumar 2009, Jebaraj et al. 2010). Sediment samples from Stn 2H (0.19 PSU salinity) produced the greatest diversity of SSU rRNA signatures based on cDNA, reflecting the presence of living *Basidiomycota* and *Ascomycota* (41 of 45 sequences). The 2 more saline sediment samples we analyzed from Stns 9H and 20H were dominated almost entirely by sequences affiliating with a much more restricted group of fungi. The nearest cultured relatives of the rRNA signatures from Stn 2H included different genera that are frequently retrieved from soils, decaying wood, stems and leaves and from leaf litter (Osono & Takeda 2007, Persson et al. 2009, Song et al. 2010). Isolates of *Westerdykella* have also been retrieved from mangrove mud (Stolk 1955), indicating that those ascomycetes are typical terrestrial/aquatic fungi. The fungal communities at Stn 2H seem to have originated from terrestrial sources but survive and grow in the freshwater environment. The dominance of *Ascomycota* in marine sediments was observed previously in the Central Indian Basin (Nagahama et al. 2001, Singh et al. 2011, 2012), contrary to a majority of *Basidiomycota* in deep-sea sediment cores of the Peru Margin and the Peru Trench (Edgcomb et al. 2011). This question of dominance may depend on sediment composition, i.e. the availability of different carbon sources (Edgcomb & Biddle 2011). However, it is quite hard to compare such studies that used different methodologies, i.e. culture-based and culture-independent methods, and also used different primers. Indeed, it has been shown that the described diversity mainly depends on the primers used (Stoeck et al. 2006). At Stn 9H, where the sediments had 1.5 PSU salinity, our clone libraries were dominated almost entirely (55 of 56 signatures) by relatives of *Nematoloma* and *Cortinarius*, basidiomycetes typically found living on dead wood. At Stn 20H, with 9.2 PSU salinity, our clone library included 57 sequences affiliated with only 3 genera: *Penicillium, Thysanophora* and *Aspergillus*. As discussed above, those species are fungi typically of terrestrial origin. At the marine Stn 20H, the fungal diversity appears lower than at Stn 2H. Such a result can be explained by the occurrence of more stringent environmental conditions (lower temperature, higher salinity and hydrostatic pressure) that likely result in fungal communities with decreasing representation from fungi of terrestrial origin.

The ITS1 rRNA analysis (DNA-based approach) supports the RNA-based and culture-based data since 3 very different sets of sequences were obtained (Table 4). Using this less-conserved genetic marker, the overall diversity revealed was lower, but the species composition was different: some basidiomycetous yeasts, 2 chytrids and 1 glomeromycete (*Scutellospora heterogama*) were revealed contrary to the SSU rRNA analysis. The ITS1 sequences we obtained were from the same types of terrestrial origins as many of the SSU rRNA sequences (phyllosphere, rhizosphere, soil, leaf litter and mangrove mud). For nearly 2 decades, eukaryotic phylogeny has been studied using SSU rRNA genes as the most common genetic marker. Recently, the ITS region was proposed as a universal DNA barcode marker for Fungi (Schoch et al. 2012) since the ITS region has the highest probability of successful identification for the broadest range of fungi. However, the suitability of the ITS region as a genetic marker is still debated due to several biases with ITS primers (Bellemain et al. 2010). Indeed, the ITS1 analysis (cDNA-based approach) produced a much more incomplete picture of diversity relative to the SSU rRNA gene in the present study (Table 5).

Fungi in the subsurface sediments seem to be more diverse than previously thought (Edgcomb et al. 2011) and could play a major role in biogeochemical cycles in the buried sediment biosphere, i.e. in denitrification processes (Cathrine & Raghukumar 2009, Mouton et al. 2012), hydrolysis and the metabolism of organic carbon.

Overlap between culture-based and culture-independent methods

Focusing on the degree to which cultured fungi appeared in the ITS1 and SSU rRNA clone libraries indicates a poor overlap between the fungi revealed using molecular vs. culture-based methods; only *Phoma* and *Trichoderma* were detected using both methods. Rarefaction curves (Fig. 3B) indicated that the sequencing effort was clearly insufficient to cover *in situ* diversity, and hence, only a fraction of the molecular diversity was revealed. Moreover, some technical biases are also inherent in culture-based studies, i.e. unculturable fungi, endophytes, cryptic

species, dormant spore germination, fast/slow growing strains, cryoconservation of sediment samples, etc., that explain this lack of overlap. Only 2 culture media were used in the present study, and fungi occurring in macro-aggregates may have difficulties growing in Petri dishes or marine broth (Damare & Raghukumar 2008). As a first investigation into the transitions within occurring and active fungal communities along a salinity gradient, the present study does indicate that fungal communities are not the same along this gradient and does provide evidence of active, truly marine or halotolerant fungi. Coupling a deeper sequencing effort and a more complex culture-based experimental plan in the future will certainly increase the overlap in fungal types detected using culture-based and molecular methods.

CONCLUSION

To determine what portions of fungi represent truly marine forms vs. inactive or active and highly adaptable, opportunistic, terrestrial organisms, we investigated fungi of the Delaware estuary. On the basis of our cultivations, microscopy (using FISH and calcofluor staining) and molecular work, we conclude that living and active fungal communities do exist in the water column and sediments of Delaware Bay, that communities in different salinity regimes are different and that there appear to be a majority of metabolically active marine fungi of terrestrial origin. Fungi may originate from terrestrial sources and be dispersed to fully marine water columns and sediments, where they are capable of surviving. The Delaware estuary community includes relatives of *Penicillium, Thysanophora* and *Aspergillus*, confirming previous studies that also showed these marine invaders in other waters (Raghukumar et al. 2004, Damare et al. 2006, Pindi 2012). In buried marine sediments, a fungal lifestyle may be advantageous, as attachment to larger physical substrates, such as buried organic material, and osmotrophy via secreted enzymes is much easier than in the water column, where needed nutrients and secreted enzymes can be lost more easily by diffusion. The mounting evidence for successful filamentous fungi and yeast forms in marine environments suggests that fungi may play a much more significant role in carbon cycling in the buried marine subsurface than previously thought. Finding a major group of halotolerant fungi in the Delaware estuary also suggests that fungi may be able to act in the marine environment, regardless of their point of origin.

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Appendix 1. Sequence-based identification of fungal strains

Station	Close neighbor	%ID	Environments	Accession no.	Source
23	*Fusarium oxysporum*	99.9	Endophytes	JF807401	Gao et al. (2011)
23	*Cryptococcus foliicola*	100	Plant leaves	AY557599	Wang et al. (2011)
23	*Tetrachaetum elegans*	99.1	Aquatic	AY357281	Belliveau & Bärlocher (2005)
23	*Leptosphaeria maculans*	99.6	Plant	LMU04238	Morales et al. (1995)
23	*Tolypocladium cylindrosporum*	99.5	Insects	AB208110	Yokoyama et al. (2006)
23	*Pochonia suchlasporia*	100	*Hevea brasiliensis*	FJ884150	Gazis & Chaverri (2010)
23	*Trichoderma citrinoviride*	100	Roots, soil, ants	HQ608144	Rodrigues et al. (2011)
23	*Phoma exigua*	100	Decaying wood	GU062320	Arhipova et al. (2011)
23	*Cadophora luteo-olivacea*	97.8	Fruits	GQ214538	Spadaro et al. (2010)
23	*Cephalosporium gramineum*	91.0	Plant	HQ322374	Wafai Baaj & Kondo (2011)
23	*Thelebolus* sp.	100	Polar environments	HQ533859	Unpublished
23	*Perisporiopsis* sp.	99.5	Endophytes	HM031458	Chaverri & Gazis (2011)
23	*Myrothecium* sp.	97.1	Plants	HQ631067	Shrestha et al. (2011)
23	*Fusarium* sp.	100	Plants	AY160209	Flowers et al. (2003)
11	*Fusarium oxysporum*	99.9	Endophytes	JF807401	Gao et al. (2011)
11	*Cystofilobasidium capitatum*	100	Soil, sediments	EU287890	Branda et al. (2009)
11	*Myrmecridium schulzeri*	99.5	Wheat straw	EU041774	Arzanlou et al. (2007)
11	*Bionectria ochroleuca*	100	Soil, plants	HQ115731	Gorfer et al. (2011)
11	*Nectria* sp.	100	Soil, rhizosphere	JF311955	Unpublished
11	*Fusarium equiseti*	100	Soil, seed, dead stem	JQ234964	Unpublished
11	*Leptosphaeria maculans*	100	Plants	LMU04238	Morales et al. (1995)
11	*Arthrinium phaeospermum*	100	Common reed	AJ279456	Wirsel et al. (2001)
11	*Verticillium nigrescens*	99.5	Plants, rhizosphere	EF543851	Zare et al. (2007)
11	*Ulocladium chartarum*	100	Soil, wood, plants	JN578634	Cueva et al. (2012)
11	*Acremonium* sp.	100	Soil, wood, plants	JN578630	Cueva et al. (2012)
11	*Cadophora luteo-olivacea*	99.3	Fruits	GQ214538	Spadaro et al. (2010)
1	*Pichia spartiniae*	99.8	Marine	FJ432595	Unpublished
1	*Kluyveromyces marxianus*	100	Marine	EU807912	Unpublished
1	*Acremonium* sp.	100	Mangrove	JN687975	Cueva et al. (2012)
1	*Rhodotorula dairenensis*	100	Soil	JN246550	Coelho et al. (2011)
1	*Coniothyrium fuckelii*	100	Endophytes, soil	AB665313	Unpublished

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Inorganic and biogenic As-sulfide precipitation at seafloor hydrothermal fields

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ABSTRACT

We investigated As-sulfides (orpiment, As_2S_3 and realgar, As_4S_4) from four seafloor hydrothermal fields: three from back-arc and one from mid-ocean ridge settings. Our studies suggest two different modes of As-sulfide precipitation at the seafloor hydrothermal sites: inorganic and biogenic. The Eh–pH modeling shows that the most stable As-sulfide at low-temperature (T < 100 °C) seafloor hydrothermal conditions is orpiment, whereas realgar appears as a stable As-sulfide phase at T = 25 °C and under a narrow range of Eh–pH values. Inorganically precipitated realgar forms euhedral crystals whereas the orpiment is mostly colloform. The biogenic type of orpiment (no realgar of this type was found) represents completely mineralized fungal hyphae. The proposed scenario of biogenic orpiment formation assumes that crack-dwelling fungal filaments in the sub-seafloor were killed by an As-rich hydrothermal fluid moving along the same crack network. The fungal organic matter served as a geochemical trap for hydrothermal As which reacted with S (leached from the basement or reduced from seawater sulfate) and mineralized the fungal hyphae as As_2S_3. This process of fungal orpiment precipitation is a mechanism of bio-sequestration of hydrothermal As and another mode of soft-tissue fossilization not described so far.

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1. Introduction

Seafloor hydrothermal input and the continental riverine runoff are the major controls of ocean chemistry (German and Von Damm, 2006). Arsenic is among the trace elements exported by the seafloor hydrothermal systems from the lithosphere to ocean in concentrations up to several hundred times those of seawater (Pichler et al., 1999; Prol-Ledesma et al., 2004; Canet et al., 2005; Leal-Acosta et al., 2010; Breuer and Pichler, 2013; Villanueva-Estrada et al., 2013). Arsenic fixation in solid phases at the submarine hydrothermal vents is essential for reducing the high hydrothermal flux of this toxic element to seawater and is an important part of the geochemical As cycle. Although the occurrence of As-sulfides at the seafloor hydrothermal sites has been reported in a number of works (Koski et al., 1988; Fouquet et al., 1993; Halbach et al., 1993; Herzig and Hannington, 1995; Nakashima et al., 1995; Burns and Percival, 2001; Petersen et al., 2002; Herzig et al., 2003; Marumo et al., 2008; Suzuki et al., 2008; Price et al., 2009; Fouquet et al., 2010) there is no careful study of these minerals to date.

Evidence shows that the seafloor hydrothermal systems at both volcanic arcs and back-arc spreading centers have As concentrations higher than those of the mid-ocean ridge systems (Fouquet et al., 1993; Douville et al., 1999; Pichler et al., 1999; Hannington et al., 2005; McCarthy et al., 2005; Price and Pichler, 2005; Breuer and Pichler, 2013) and this is ascribed to seawater–acidic rock interaction (Douville et al., 1999). Therefore, these settings appear to be the most prominent sites for investigation of As-sulfide precipitation. Motivated by the virtual lack of investigations of the seafloor hydrothermal As-sulfides, we explored three recent seafloor hydrothermal fields from volcanic arc–back-arc setting in the western Pacific Ocean (Fig. 1A): JADE, Kaia Natai Seamount and Conical Seamount; and one from mid-ocean ridge: Mt. Jourdanne. Here we report on the results of a mineralogical and geochemical study of these minerals.

2. Geologic setting

JADE hydrothermal field lies in the tectonically active intra-continental Okinawa Trough, a back-arc setting related to the

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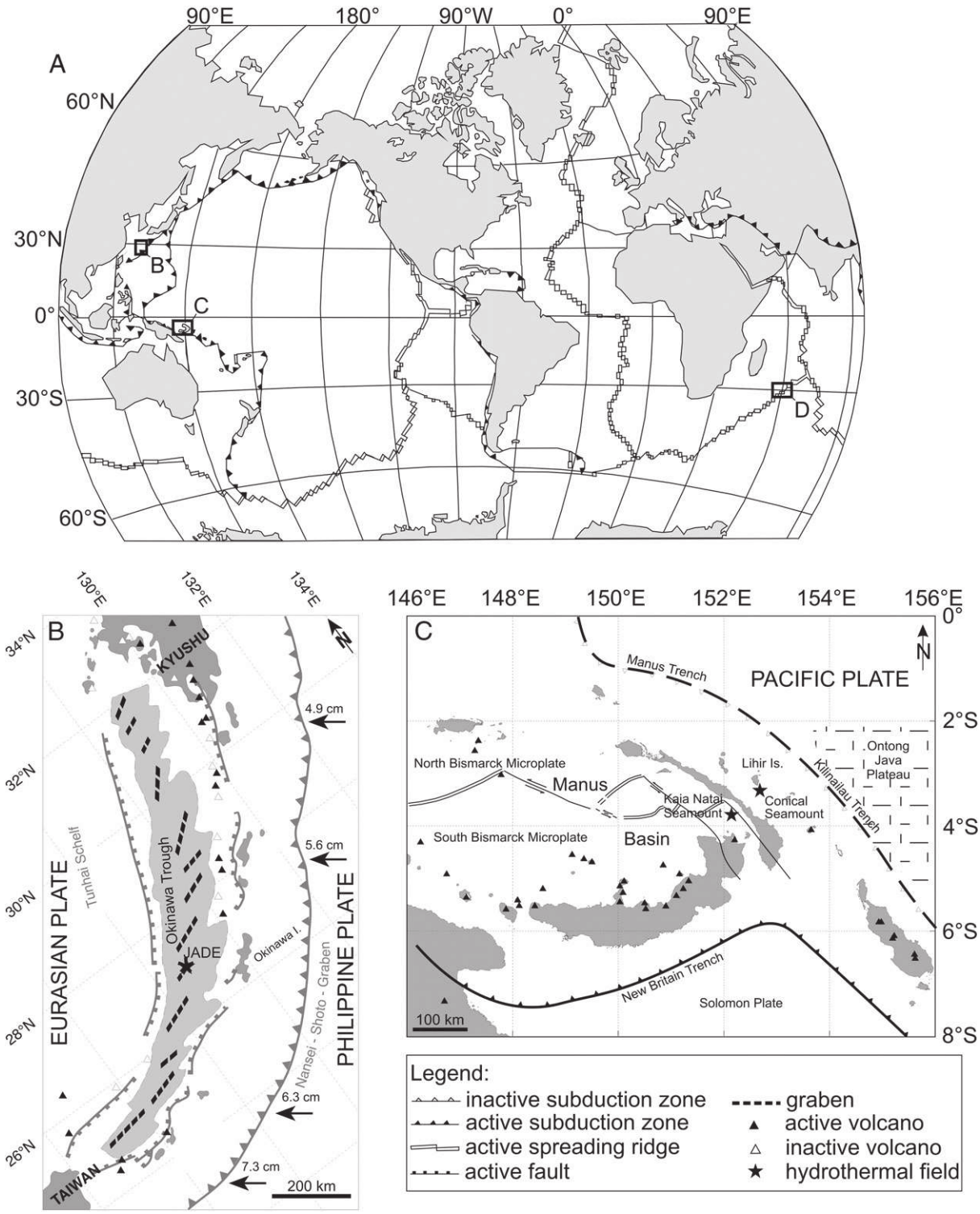

Fig. 1. (A) Schematic map of the Ocean with the areas (boxes) where As-sulfides have been sampled for this study; (B) map of the Okinawa Trough with JADE hydrothermal field; (C) map of the Manus back-arc basin and Manus–Kilinailau arc–trench system with Kaia Natai and Conical Seamounts hydrothermal fields, respectively; D on A shows the position of Mt. Jourdanne at the SWIR.

subduction of the Philippine Plate under the Eurasian Plate (Fig. 1B). Previous studies indicated that active back-arc spreading existed in the southwest part of the trough, whereas the northeastern part of the trough is still in the rifting phase characterized by normal faulting, incipient crustal extension and subsidence (Halbach et al., 1993). The thickness of the continental crust ranges from 18 km in the south to ~30 km in the north (Hirata et al., 1991). The rate of relative motion of the Philippine Plate with respect to the Eurasian Plate increases from northeast to southwest (Seno, 1977). In the middle Okinawa Trough, back-arc volcanism forms a series of parallel elongated ridges within graben segments (Sibuet et al., 1987). These ridges are composed of a variety of young volcanic rocks: rhyolites, dacites, andesites

Table 1
Investigated As-sulfides.

Sample #	Locality	Setting	Latitude	Longitude	Depth (m)	Sampling device	Hand sample description	Mineralogy
MN-DR-1-11	Kaia Natai Smt., East Manus Basin	Intra-oceanic back-arc spreading center	03°49.60′S	152°11.54′E	1401	Dredge	Volcanic breccias: altered and unaltered angular to subangular fragments, pyritized fragments. Red-stained cracks.	Orpiment
SO-133 25GTVA-7	Conical Smt., Tabar–Feni Island chain	Fore-arc with extension related to subduction reversal	03°18.728′S	152°39.543′E	1093	TV-grab	Intensely altered and silicified ankaramitic basalt with disseminated sulfides including As-sulfides.	Realgar, orpiment
SO-166 43RD (0–5 cm)	- " -	- " -	03°18.720′S	152°39.535′E	1084	BGS Rockdrill	Intensely silicified ankaramitic basalt with disseminated sulfides.	Realgar, orpiment
SO-56 159DSE; SO-56 160DSE	JADE field, Okinawa Trough	Intra-continental back-arc spreading center	27°17′06″N	127°04′40″E	1350	Dredge	Outer portions of black smokers (rim), dominated by lower-temperature sulfides (As, Sb, Hg) in a matrix of barite and amorphous silica (opal).	Realgar, orpiment, silica, barite
454 No 6a/b	Mt. Jourdanne, Southwest Indian Ridge	Mid-ocean ridge	27°50.90′S	63°56.20′E	2940	Submersible Shinkai (6500)	Sulfide mineralizations (together with barite) and cementations of brecciated and altered basalt.	Realgar, barite

and basalts (Kimura et al., 1988; Halbach et al., 1993; Shinjo and Kato, 2000). The JADE hydrothermal field (Fig. 1B) is located at the northeastern slope of the Izena depression, which was formed by a strike–slip strain accompanying the rifting of the two overlapping graben segments in this area (Halbach et al., 1993). It is composed of active and inactive sulfide–sulfate chimneys and mounds.

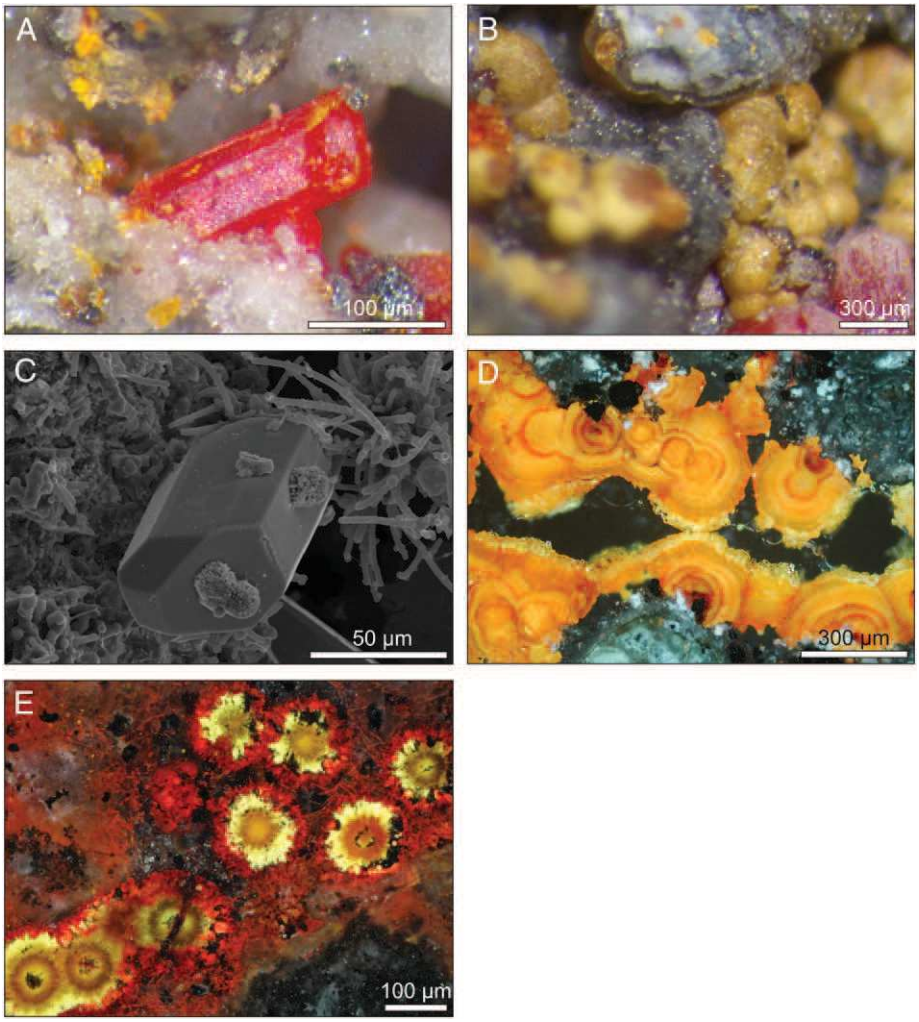

Fig. 2. Photomicrographs of: (A) euhedral realgar (red) surrounded by amorphous silica (white) (sample #SO-56 159DSE; stereo-microscope); (B) colloform orpiment (grayish-yellow) and euhedral realgar (red) (sample #SO-133 25GTVA-7; stereo-microscope); (C) realgar crystal surrounded by filamentous (bacteria-like) amorphous silica (sample #SO-133 25GTVA-7; SEM SEI); (D) colloform orpiment (orange-yellow) [sample #SO-166 43RD (0–5 cm); optical polarizing microscope, reflected light, XN]; (E) radial As-sulfides (mainly orpiment) in a siliceous matrix from a black smoker rim (sample #SO-56 160DSE; optical polarizing microscope, reflected light, XN); note the small filamentous red aggregates in the upper right part of the picture, which could not further be analyzed, because they were embedded in resin.

Kaia Natai Seamount (Manus Basin; Fig. 1C) is situated in an intra-oceanic back-arc spreading setting. Manus Basin is structurally bounded by both the inactive Manus and active New Britain subduction zones and floored by North and South Bismarck microplates (Fig. 1C). The active spreading in the Manus Basin occurs at three successive ridge segments offset by Willaumez, Djaul and Weitin transform faults (from west to east; Fig. 1C). Ongoing hydrothermal activity has been documented on the Manus spreading center and in the East Manus Basin (Binns and Scott, 1993; Gamo et al., 1997; Auzende et al., 2000; Craddock et al., 2010; Reeves et al., 2011). The East Manus Basin (between the Djaul and Weitin transform faults) is an active transform zone within island-arc crust formed during previous subduction of the Pacific Plate under the New Ireland Arc (Taylor, 1979; Binns and Scott, 1993; Martinez and Taylor, 1996). Magmatic activity associated with the incipient rifting of felsic crust has produced a series of volcanic ridges composed of a wide range of lavas (from basalts to rhyodacites) showing strong arc affinities (Binns and Scott, 1993; Sinton et al., 2003). Kaia Natai is a submarine volcano situated east of the well-studied active SuSu Knolls fields and hosts an inactive hydrothermal field that covers its summit and upper part of the southeastern slope (Auzende et al., 2000).

Conical Seamount lies on the Tabar–Feni Island chain (Fig. 1C), which is a fore-arc subjected to extension related to subduction reversal. In the Early Miocene, this region was dominated by westward subduction of the Pacific Plate beneath the New Ireland Arc. Between 24 and 11 Ma, this subduction was blocked by the collision of the Ontong Java Plateau with the trench (Coleman and Kroenke, 1981). Conical Seamount is located south of the Lihir Island (Fig. 1C) and consists of a simple cone with a small summit plateau. Conical Seamount is composed of high-K calc-alkaline to shoshonitic, pyroxene-phyric trachybasalt (Petersen et al., 2002). Au-rich, siliceous veins with disseminated polymetallic sulfides and pyritic stockwork mineralization have been recovered from the top of the seamount (Petersen et al., 2002).

A well-defined neovolcanic ridge (Mt. Jourdanne), is located at the Southwest Indian Ridge (SWIR) west of the Rodriguez Triple Junction (Fig. 1A) (Münch et al., 1998, 2001). The seafloor here displays several types of basaltic lavas: shallower slopes are dominated by sheet flows (lobate, folded), which are often covered by thin sediments, whereas the summit is dominated by pillows and tubes. The summit of the ridge is cut by an E–W trending graben (~100 m wide), accompanied by several smaller fissures running both parallel and perpendicular to the graben. Here, an inactive hydrothermal field focuses on these structures. The mineralogy of the collected samples, which is unusual for this geologic setting, includes chalcopyrite, pyrrhotite, sphalerite, pyrite, galena, Pb–As sulfosalts, realgar, boulangerite, and barite.

3. Material and methods

We studied rock and sulfide–sulfate samples containing As-sulfides from the aforementioned seafloor hydrothermal fields (Table 1). Four of the samples (two host-rocks and two sulfide–sulfates) were collected from the seafloor surface whereas one (host-rock) was recovered from the sub-surface with the British Geological Survey (BGS) Rockdrill (Petersen et al., 2005). After preliminary investigation with stereo-microscope (WILD M8), specimens from all samples were prepared as both polished and thin sections and investigated with Olympus BX60 polarizing microscope.

Secondary electron images (SEI) and energy dispersive X-ray spectra (EDS) were obtained on small (~0.5 × 0.5 cm) sub-samples, mounted on aluminum stubs using carbon tape and coated with Au using a FEI Quanta 200 scanning electron microscope (SEM) (V = 10 keV, I = 100 μA, electron beam diameter of 2 μm). EDS data were also gathered from C-coated polished sections using a Zeiss SEM (DSM 960A; 25 keV, beam diameter 5 μm). X-ray mapping [in As L_α, Ba L_α, Zn K_α and S K_α lines and back-scattered electrons (BSE)] and contents of elements with $Z \geq 9$ were acquired on C-coated polished sections using Cameca SX 100 electron microprobe (EMP) (V = 15 keV, I = 20 nA, electron beam diameter of 2 μm).

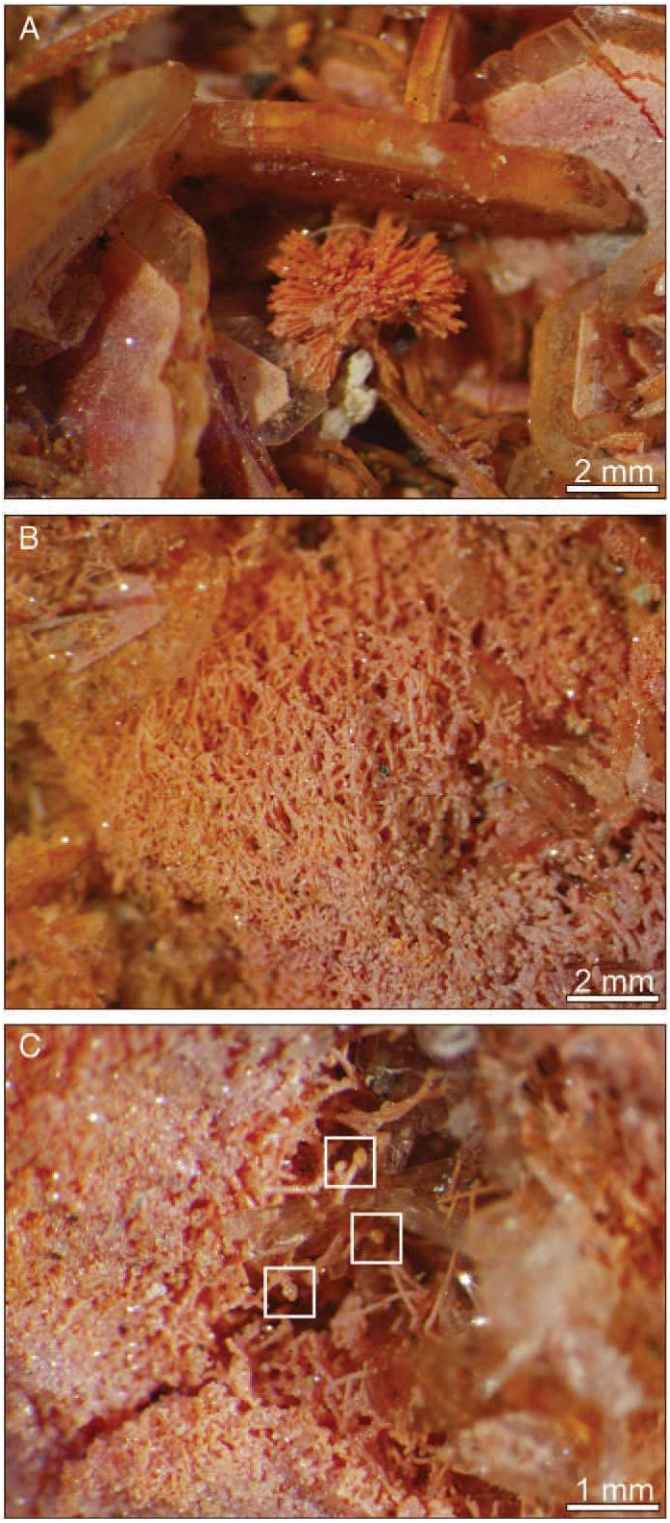

Fig. 3. Photomicrographs (sample #MN-DR-1-11; stereo-microscope) of orpiment filaments: (A) tight cluster of filaments among euhedral barite crystals; (B) dense network of filaments; (C) filaments with conidiophores and spores (white squares).

Table 2
Lattice parameters (mean values) of investigated As-sulfide crystals.

Sample #	Mineral	*a* (Å)	*b* (Å)	*c* (Å)	*β* (°)	*V* (Å^3)
MN-DR-1-11 (1)[a]	Orpiment	11.473 (5)	9.581 (4)	4.256 (2)	90.69 (4)	467.8 (3)
SO-133 25GTVA-7 (3)[a]	Realgar	9.325 (1)	13.570 (1)	6.590 (1)	106.37 (1)	800.1 (1)
SO-56 159DSE (2)[a]	Realgar	9.325 (1)	13.570 (1)	6.589 (1)	106.38 (1)	800.0 (1)

[a] Number of investigated crystals.

Standards used were: FeS_2 (S K_α and Fe K_α), Cu metal (Cu K_α), Sb_2S_3 (Sb L_α), ZnS (Zn K_α), HgS (Hg L_α), Ag metal (Ag L_α) and GaAs (As L_α).

The unit-cell parameters of selected realgar single crystals [three from Conical Seamount (#SO-133 25GTVA-7) and two from JADE hydrothermal field (#SO-56 159DSE)] were obtained by means of an Oxford Diffraction Excalibur^{III} X-ray single-crystal diffractometer using graphite-monochromatized MoK_α radiation.

As filamentous orpiment (#MN-DR-1-11; Kaia Natai Seamount) did not diffract as a single crystal (we observed only diffraction rings and no diffraction spots) and the amount of available material was scarce, powder diffraction data were collected using an Oxford Diffraction Excalibur PX Ultra single-crystal diffractometer (CuK_α radiation) with a 165-mm diagonal Onyx charge-coupled device detector at 2.5:1 demagnification. The program *Crysalis* RED (Oxford Diffraction, 2006) was used to convert the observed diffraction rings into a conventional X-ray diffraction (XRD) pattern.

Eh–pH diagrams of As–S phases were produced using the Geochemist's Workbench 8.0 software and the "thermo_minteq" database. Physical and chemical parameters used in our calculations are discussed in Section 5.

In order to avoid introduction of exogenous contaminants in the molecular analyses of the filamentous orpiment sample (#MN-DR-1-11) it was sub-sampled with stainless tweezers in sterile Eppendorff® tubes, not touched with ungloved hands. We processed DNA extraction and purification from 2 × 0.5 g of inert filamentous orpiment (#MN-DR-1-11) using MoBio PowerSoil DNA isolation and purification kits following manufacturer's instructions. Negative DNA extraction control was processed. Concentration of extracted DNA was measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and nested-PCR assays were performed to amplify ITS1 region of fungal rRNA using ITS1F, ITS4 and ITS2 primers as described elsewhere (Burgaud et al., 2013). All PCR mixtures contained 1× FastStart *Taq* DNA polymerase buffer with $MgCl_2$ (2 mM) (Roche), 1 mM of additional $MgCl_2$, 240 μM dNTP, 0.4 μM of each primer, 1 unit of FastStart *Taq* DNA polymerase (Roche) and 1 μL of DNA template. Positive and negative controls were prepared. Amplicons size (between 250 and 300 bp) was checked on 1% agarose gel using 1 kb-DNA ladder (Promega). ITS rRNA fragments were cloned into the TOPO TA cloning vector pCR2.1 and transformed in TOP10 chemically competent *Escherichia coli* according to manufacturer's instructions (Invitrogen). Transformants were selected on Luria–Bertani (LB) agar plates containing ampicillin (100 μg/mL). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F and M13R. Clones were then sequenced by GATC Biotech and inserts were analyzed using DNA Baser Sequence Assembler (Heracle Biosoft) and identify using a blastn search in the NCBI database. We also stained thin sections of filamentous orpiment on slides with Calcofluor White M2R {4,4′-bis[4-anilino-6-bis(2-ethyl)amino-s-triazin-2-ylamino]-2, 2′-disulfonic acid}. The targets of calcofluor white M2R are chitin, cellulose, and carboxylated polysaccharides. Following incubation in the dark for 5 min of filamentous orpiment and positive control (fixed fungal cells), slides were washed with sterile water and observed using epifluorescence microscopy (Zeiss Axioplan 2 imaging microscope equipped with a Zeiss AxioCam camera).

4. Results

The collected As-sulfides show three different morphologies: euhedral crystals (Fig. 2A), colloform aggregates (Fig. 2B) and dense networks of filaments (Fig. 3).

Red euhedral crystals precipitated at the seafloor (samples #SO-133 25GTVA-7 and #SO-56 159DSE) and sub-seafloor [sample #SO-166 43RD (0–5 cm)] appeared to be realgar (XRD and EMP data): As_4S_4. Realgar forms prismatic crystals scattered in a matrix of colloform and filamentous X-ray amorphous silica ($SiO_{2am.}$) (Fig. 2A, C), barite and stibnite. Its diffraction peaks at the XRD patterns (not presented) were sharp and strong. Realgar unit-cell parameters, $a = 9.324–9.326$ (1) Å, $b = 13.568–13.571$ (1) Å, $c = 6.588–6.591$ (1) Å, $\beta = 106.35–106.40$ (1)°, $V = 799.8–800.2$ (1) Å^3 (Table 2) are in excellent agreement with those of synthetic As_4S_4 (Mullen and Nowacki, 1972). It contains traces of Sb, Zn and Ag, and has a structural formula close to the stoichiometry: $As_{3.98}Sb_{0.02}Zn_{0.01}S_{3.99}$ (Table 3).

Grayish-yellow to orange-yellow orpiment (As_2S_3; Fig. 2B) forms colloform aggregates in the voids of the host rocks at and beneath the seafloor [samples #SO-133 25GTVA-7, #SO-166 43RD (0–5 cm) and #SO-56 159DSE]. In cross-section it shows concentric zonation (Figs 2D; 4A, B). It is often coated by a thin film of sphalerite (Fig. 4B, C). This orpiment has traces of Zn, Sb and Ag, and a structural formula close to the stoichiometry: $As_{1.96}Zn_{0.02}S_{3.01}$ (Table 3).

Table 3
Chemical composition (EMP and EDS data; the latter for SO-56 samples) and structural formulae (on the basis of 5 and 8 atoms for orpiment and realgar, respectively) of investigated As-sulfides.

Sample #	Mineral	As (wt.%)	S (wt.%)	Sb (wt.%)	Zn (wt.%)	Fe (wt.%)	Cu (wt.%)	Hg (wt.%)	Ag (wt.%)	Total (wt.%)	Structural formulae								
											As	S	Sb	Zn	Fe	Cu	Hg	Ag	Total
MN-DR-1-11 (8)[a]	Orpiment	57.49	42.05	0.05	0.14	0.02	0.03	0.15	–	99.92	1.84	3.15	0.00	0.01	0.00	0.00	0.00	–	5
SO-133 25GTVA-7 (9)[a]	Orpiment	58.96	38.90	0.14	0.59	–	–	–	0.12	98.72	1.96	3.01	0.00	0.02	–	–	–	0.00	5
SO-133 25GTVA-7 (63)[a]	Realgar	69.65	29.88	0.63	0.09	–	–	–	0.06	100.31	3.98	3.99	0.02	0.01	–	–	–	0.00	8
SO-56 160DSE (34)[a]	Realgar	71.50	28.50	–	–	–	–	–	–	100.00	4.14	3.86	–	–	–	–	–	–	8
SO-56 160DSE (20)[a]	Orpiment	59.93	40.07	–	–	–	–	–	–	100.00	1.95	3.05	–	–	–	–	–	–	5

[a] Number of point analyses.

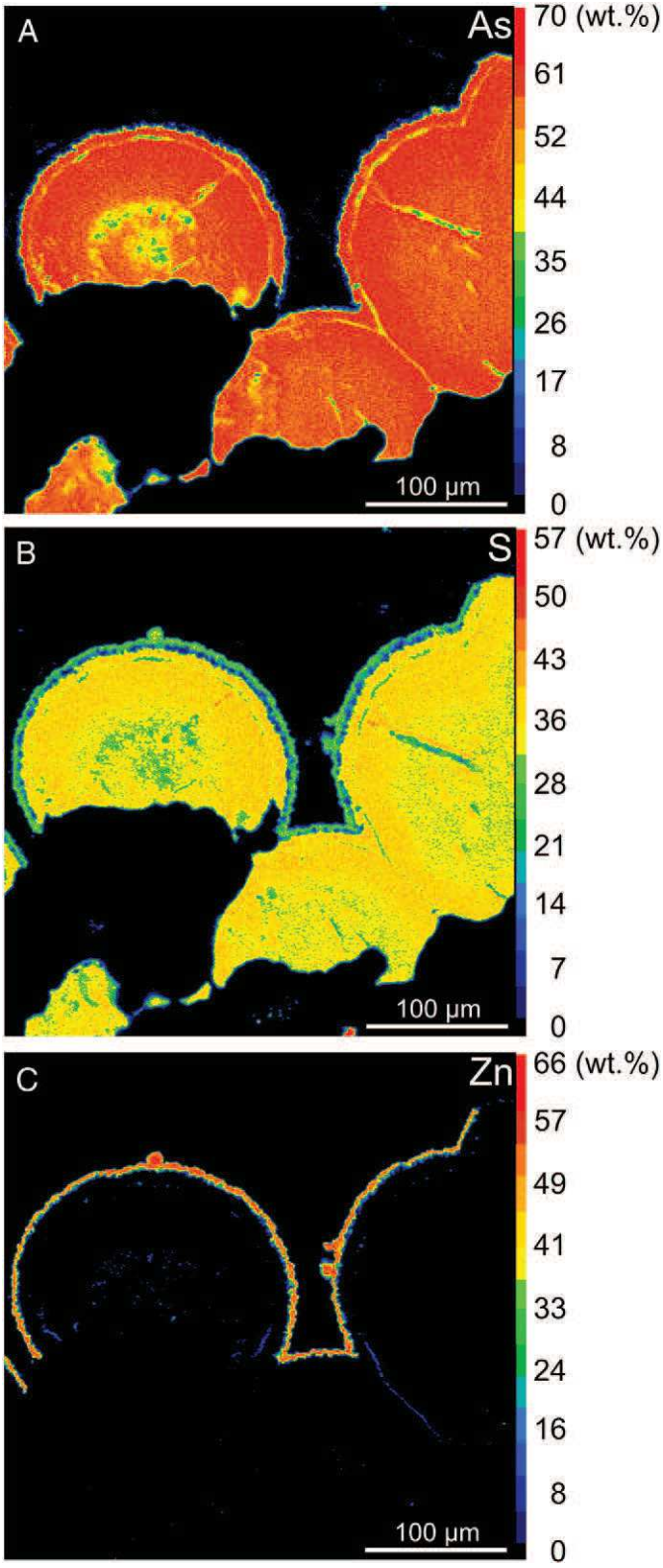

Fig. 4. X-ray maps of colloform orpiment (sample #SO-133 25GTVA-7; polished section): (A) X-ray scan in As L_α; (B) X-ray scan in S K_α; (C) X-ray scan in Zn K_α. Color scales (right-hand side), elemental concentrations.

Radiating orpiment aggregates (#SO-56 DSE160; Fig. 2E) displaying color variations from yellow to orange to red did not reveal significant or consistent compositional differences (Table 3) in relation to the respective color.

Orange to red filamentous orpiment (sample #MN-DR-1-11) fills cracks in the host rocks close to the seafloor. It forms flower-like clusters (Fig. 3A) and dense networks (Fig. 3B, C) among barite crystals. Filaments fashion a complex mycelium-like network showing filamentous fungal elements. The flower-like clusters of filaments (Fig. 3A) appeared like fungal sporodochia. Some filaments bear spore-like elements (Fig. 3C), which might be interpreted as conidiophores. Accurate observation of the filamentous network (using SEM) reveals small filaments growing perpendicular to the surface of the crack wall with a tapered extension zone of 15 to 20 μm-diameter (Fig. 5A). Cross walls (septa) were observed along the filaments (Fig. 5B). Other interesting features were the rough-walled filaments (Fig. 5C) and blastic-retrogressive conidiogenesis (Fig. 5D). The latter is a process in which first conidia are formed at the tip of conidiogenous hypha and other conidia are shaped just beneath the first ones. This kind of conidiogenesis is typical for *Dikarya* and more precisely ascomycetous fungi. The filaments have concentric internal structure composed of four layers (Fig. 6A). They are impregnated by As-sulfide (Fig. 6B, C) and develop in barite matrix (Fig. 6D). No interesting results were collected from DNA extraction and sequencing since only typical aerial fungal contaminants were obtained, but not DNA from the filamentous orpiment. No results were obtained from chitin staining. XRD studies (Table 4) proved that these filaments are composed of orpiment. The refined unit-cell parameters of this orpiment are: $a = 11.4731$ (5) Å, $b = 9.5807$ (4) Å, $c = 4.2562$ (2) Å, $\beta = 90.692$ (4)°, $V = 467.81$ (3) Å^3 (Table 2). Orpiment contains traces of Hg, Zn, Sb, Cu and Fe, and has a structural formula with excess S: $As_{1.84}Zn_{0.01}S_{3.15}$ (Table 3).

5. Discussion

The results of our investigation imply two different mechanisms of As-sulfide precipitation: inorganic (realgar and colloform orpiment) and biogenic (filamentous orpiment). The occurrence of realgar and orpiment in close association with hydrothermal minerals (sulfides, sulfates, silicates) at active seafloor hydrothermal sites suggests they are genetically linked to hydrothermal As input.

In order to constrain the formation conditions of the realgar and colloform orpiment mineralization, we modeled Eh–pH phase diagrams for the As–S system (Fig. 7). Since we have no data for the hydrothermal fluids from any of the four hydrothermal sites studied here, we used the physical–chemical parameters of the fluids venting at the Ambitle Island seafloor field (Price et al., 2007). This field occurs in the same geological setting as the Conical Seamount mineralization (Tabar–Feni Island chain) and therefore, the conditions we used were: T = 100–25 °C, P = 120 bars, [As] = 900–1000 μg/L, and $[SO_4]$ = 900–1900 mg/L. We show here (Fig. 7) calculations only for 25 and 100 °C, however from looking also at intermediate temperatures we find that in the temperature range from 25 to 100 °C orpiment is the most dominant As mineral phase, showing stability in a wide pH range (Fig. 7A). Realgar, on the other hand, appears as a stable As-sulfide phase only when T ≈ 25 °C and under a narrow range of Eh–pH values (Fig. 7B). Data from Eh–pH modeling of JADE hydrothermal fluids (Pracejus et al., 1997) support this finding and indeed place the observed realgar into a very narrow stability field (Eh ~ 153–140 mV; pH ~ 4.8–5.0; T < 100 °C). This means that the most stable As-sulfide at low-temperature (T < 100 °C) seafloor hydrothermal conditions is orpiment.

The presence of tight clusters of filaments (putative sporodochia), rough-walled filaments, septa and blastic-retrogressive conidiogenesis within the filamentous orpiment are typical characteristic features of fungal organism. Therefore, we consider that this type of orpiment represents completely mineralized hyphae and explains why no DNA from filamentous orpiment was successfully extracted and chitin visualized.

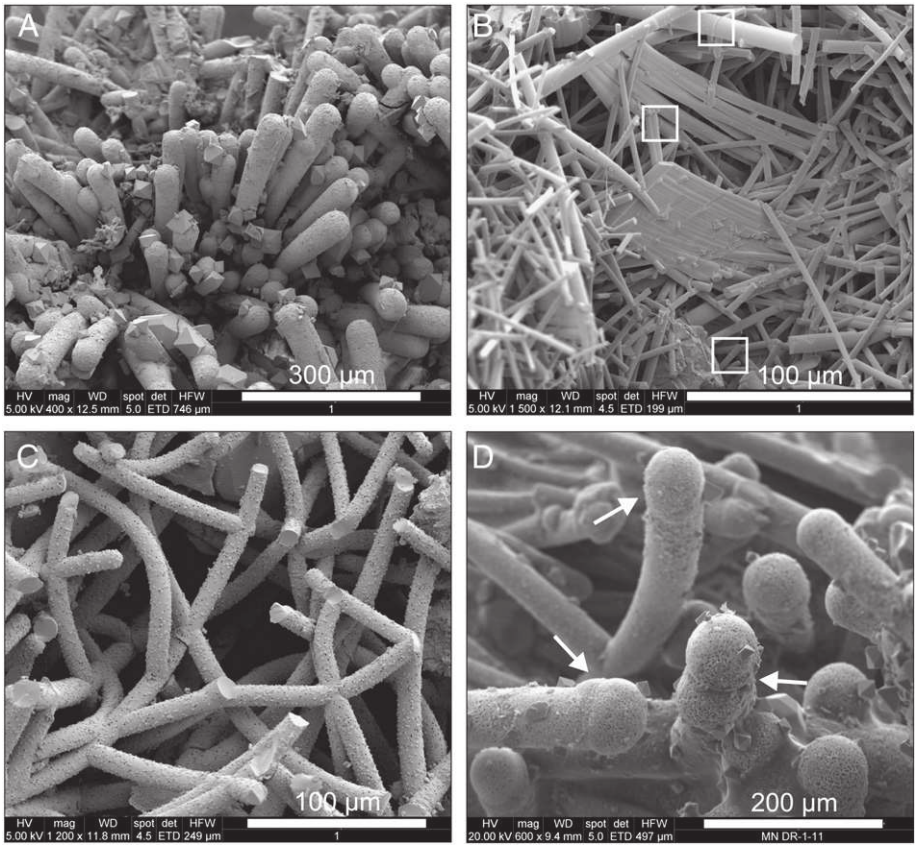

Fig. 5. SEM photomicrographs (sample #MN-DR-1-11; SEI) of fungal elements: (A) putative sporodochia; (B) network of filaments with septa (white squares); (C) rough-walled filaments with branching; (D) fungal conidiogenesis (white arrows).

We suppose that there are two alternative mechanisms of orpiment mineralization of the fungi: (1) fungi have thrived in a sub-seafloor hydrothermal environment with high As flux, precipitated As_2S_3 intra- and/or extra-hyphal during their metabolism and eventually got mineralized; (2) fungi have lived in As-free sub-seafloor environment, have been killed and subsequently mineralized

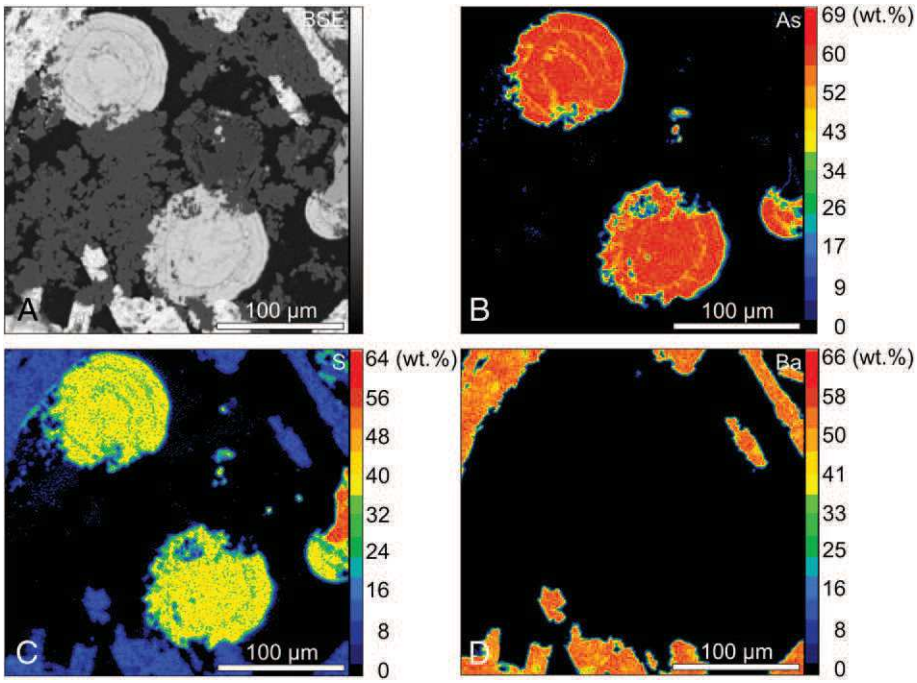

Fig. 6. BSE image and X-ray maps of cross-cut fungal elements among barite crystals (sample #MN-DR-1-11; polished section): (A) BSE image; (B) X-ray scan in As L_α; (C) X-ray scan in S K_α; (D) X-ray scan in Ba L_α. Color scales (right-hand side), elemental concentrations.

Table 4
X-ray powder diffraction pattern of filamentous As-sulfides (#MN-DR-1-11).

d_{meas}	d_{calc}	I/I_0	h	k	l
7.35	7.3536	15	1	1	0
4.92	4.9215	15	2	1	0
4.79	4.7904	100	0	2	0
4.42	4.4205	5	1	2	0
4.01	4.0060	5	−1	0	1
3.97	3.9746	30	1	0	1
3.70	3.6959	40	−1	1	1
3.67	3.6712	5	1	1	1
3.55	3.5516	5	3	1	0
3.18	3.1816	35	0	2	1
3.08	3.0766	10	1	3	0
3.06	3.0588	15	1	2	1
2.989	2.9886	10	3	2	0
2.868	2.8681	5	4	0	0
2.862	2.8617	45	−3	0	1
2.793	2.7930	15	−2	2	1
2.791	2.7903	10	2	3	0
2.772	2.7717	10	2	2	1
2.742	2.7420	5	−3	1	1
2.712	2.7119	65	3	1	1
2.555	2.5544	15	0	3	1
2.462	2.4607	10	4	2	0
2.456	2.4567	20	−3	2	1
2.452	2.4512	25	3	3	0
2.344	2.3446	5	1	4	0
2.321	2.3206	10	−4	1	1
2.296	2.2963	5	4	1	1
2.231	2.2314	5	5	1	0
2.140	2.1399	10	−4	2	1
2.121	2.1208	10	4	2	1
2.087	2.0873	15	0	4	1
2.077	2.0773	15	0	1	2
2.069	2.0693	5	5	2	0
2.029	2.0299	10	3	4	0
1.914	1.9144	10	−4	3	1
1.902	1.9007	5	4	3	1
1.875	1.8751	5	6	1	0
1.863	1.8634	5	5	3	0
1.753	1.7528	5	−1	3	2
1.747	1.7472	20	0	5	1
1.741	1.7412	5	−3	2	2
1.713	1.7131	10	3	5	0
1.692	1.6919	15	−4	1	2
1.687	1.6873	5	2	3	2
1.683	1.6829	5	4	4	1
1.673	1.6730	10	4	1	2

by hydrothermal As flux. We are discussing them in further detail below.

Life in the sub-seafloor is highly structured and composed of diverse and complex active communities from subsurface down to depths of more than 1000 m below the seafloor, as pinpointed by molecular methods (Schippers et al., 2005; Biddle et al., 2011; Morono et al., 2011; Orcutt et al., 2011). For years, focus was concentrated on prokaryotes, but recently different studies assessed the occurrence and activity of microeukaryotes and especially fungi in the sub-seafloor (Schumann et al., 2004; Schippers and Neretin, 2006; Edgcomb et al., 2011; Ivarsson, 2012; Ivarsson et al., 2012). Fungi obviously play an important role in biogeochemical cycles with the recycling of nutrients and weathering of minerals.

It has been found that a number of bacterial species precipitate As-sulfides both intra- and extracellularly (Newman et al., 1997; Demergasso et al., 2007; Ledbetter et al., 2007; Jiang et al., 2009). This biomineralization of a toxic compound is intimately tied to bacterial metabolism (Newman et al., 1997). Some terrestrial fungi are used in bioremediation through their As biosorption and/or biovolatilization abilities (Cernansky et al., 2007). Others are able to bioaccumulate low concentrations of arsenopyrite (FeAsS) in their biomass (Adeyemi, 2009). However, even the hyper-tolerant fungi are only able to grow at arsenate concentrations of 0.2 M (Canovas et al., 2003). Therefore, even now we do not have unambiguous proof that the fungi dwell in As-rich environments and may rule out the first mechanism of orpiment mineralization of the fungi.

The second mechanism we propose (Fig. 8), assumes that the fungal filaments inhabiting cracks of the seafloor basement (stage 1) have been killed by an As-rich hydrothermal wave moving along the same crack network (stage 2) from the reaction zone where the fluid has formed to the seafloor. Fungi are composed of organic matter. Organic matter has been found to be a major sorbent for As in S-rich anoxic environments and it can completely sequester As through the formation of covalent bonds between As^{3+} and organic S groups (Langner et al., 2012). By binding As in this way, organic matter plays an active role in As immobilization in S-enriched, anoxic environments. Under reducing conditions organic matter triggers the formation of As-sequestering sulfides, leading to a reduction in As mobility (Langner et al., 2012). Whereas As in the seafloor hydrothermal fluids is supposed to be leached from the basement rocks and eventually enriched through fluid phase separation (Douville et al., 1999) the reactive S in these fluids close to the seafloor may have dual origin: (1) leached from the rocks and transported as H_2S in the fluids; (2) product of seawater SO_4^{2-} reduction by bacteria inhabiting the same sub-seafloor crack network. Eventually, the fungal organic matter likely serves as a geochemical trap for hydrothermal As which might react with S (leached from the basement or reduced from seawater sulfate) and mineralize fungal hyphae as As_2S_3. This second mechanism appeared consistent with the absence of extracted DNA and chitin visualization.

This process of fungal orpiment precipitation has several important implications. First, this is a mechanism of bio-sequestration of a highly-toxic element like As supplied to the ocean by seafloor hydrothermal systems and the sub-seafloor crack network inhabited by fungi acts as an As filter. Second, As-sulfide mineralization of organisms without mineral skeletal elements (e.g., fungal hyphae) is another mode of soft-tissue fossilization not described so far (Schopf, 1975; Brasier et al., 2011; Cai et al., 2012). Third, this occurrence supports the previous observations of fungal inhabitation of the deep sub-seafloor (e.g., Raghukumar et al., 2004; Ivarsson et al., 2012) and suggests that their presence and roles in these untapped paleoecosystems have been clearly underestimated.

Organisms have developed strategies through which they volatilize and mineralize toxic compounds in their aquatic environment (White et al., 1995; Pracejus and Halbach, 1996). This reduces the activity/concentration of the respective toxic compound in the surrounding environment, even if the individual organism eventually dies. Although the microorganisms thrive at seafloor hydrothermal environment they have also to survive at high levels of dissolved toxic heavy metals (Ag, As, Cd, Cu, Hg, Pb, and Zn, plus S^{2-}) in the vent fluids. A number of marine organisms can accumulate different heavy metals to levels of ten times those of the background concentrations without being harmed (Francesconi and Edmonds, 1998). However, once the threshold for an element is reached, these organisms need to detoxify their system (effectively "cleaning" their environment), otherwise these seafloor "oases" would not be able to sustain the entire food chain from microbes to fishes over a long time-span. Microbially precipitated minerals can act as nucleation sites for further heavy metal accumulation (e.g., cinnabar filaments at the JADE hydrothermal field; Pracejus and Halbach, 1996). Cell walls enriched in certain compounds may also act as starting point for further mineral precipitation, once an initial crystallization nucleus has been formed (Sağlam et al., 1999; Das et al., 2008). Organic molecules with charged terminations on the outer cell membrane could act as nucleation site as well. Arsenic that enters the cell by diffusion through its outer membrane (Oremland and Stolz, 2003; Hub and de Groot, 2008) is converted into organo-As compounds, which can then be excreted more easily by the organism (e.g., Hg; Pracejus

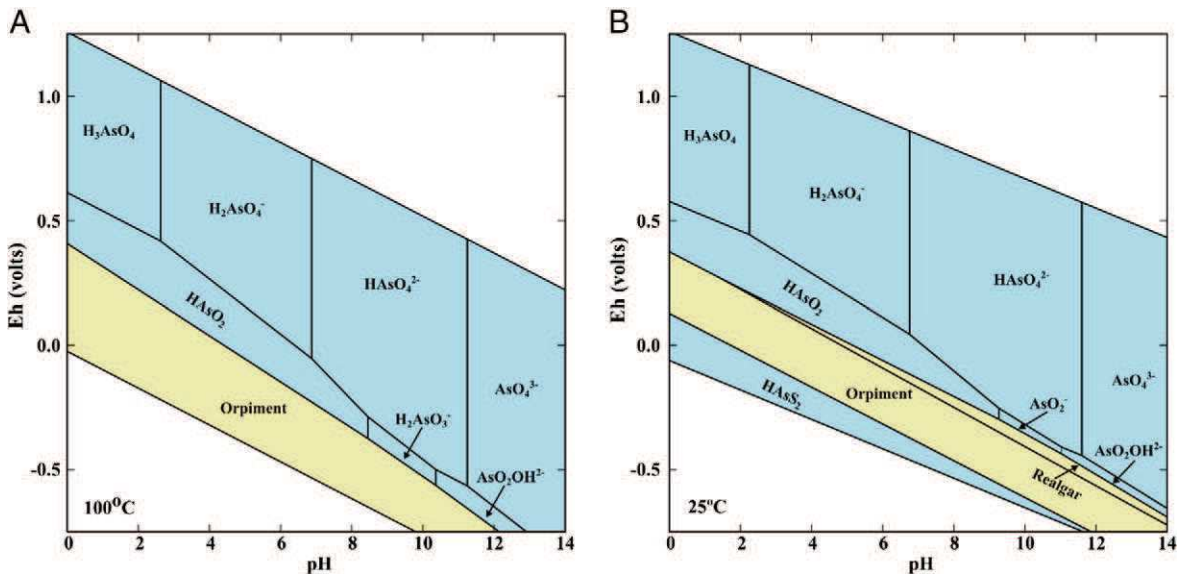

Fig. 7. Eh–pH phase diagrams for the As–S system calculated at P = 120 bars, [As] = 900–1000 μg/L, and [SO_4] = 900–1900 mg/L. (A) stability fields of As fluid speciation and minerals at T = 100 °C; (B) stability fields of As fluid speciation and minerals at T = 25 °C. Yellow fields indicate mineral phases and blue fields indicate dissolved species.

and Halbach, 1996) or accumulated in the cell (Francesconi and Edmonds, 1998).

6. Summary

As-sulfides, orpiment and realgar, appear to be minor minerals at seafloor back-arc hydrothermal fields and very rare at mid-ocean ridge hydrothermal sites. According to their mode of precipitation they are inorganic or biogenic. Inorganic orpiment and realgar form colloform aggregates and euhedral crystals, respectively. They both have composition close to the stoichiometry. Filamentous orpiment shows typical features of fungi and, therefore, we consider that it represents completely mineralized fungal hyphae. No biogenic realgar was found. Our hypothesis for biogenic orpiment precipitation assumes that an As-containing hydrothermal fluid moving through the cracks of the seafloor basement has killed the fungal colonies living in the sub-seafloor. The fungal organic matter served as a geochemical trap for hydrothermal As which reacted with S (leached from the basement, reduced from seawater sulfate or provided through S-bearing magmatic volatiles) and mineralized fungal hyphae as As_2S_3. The most important implications of this process are that this is a mechanism of bio-sequestration of hydrothermal As and that As-sulfide mineralization of organisms without mineral skeletal elements is another mode of soft-tissue fossilization.

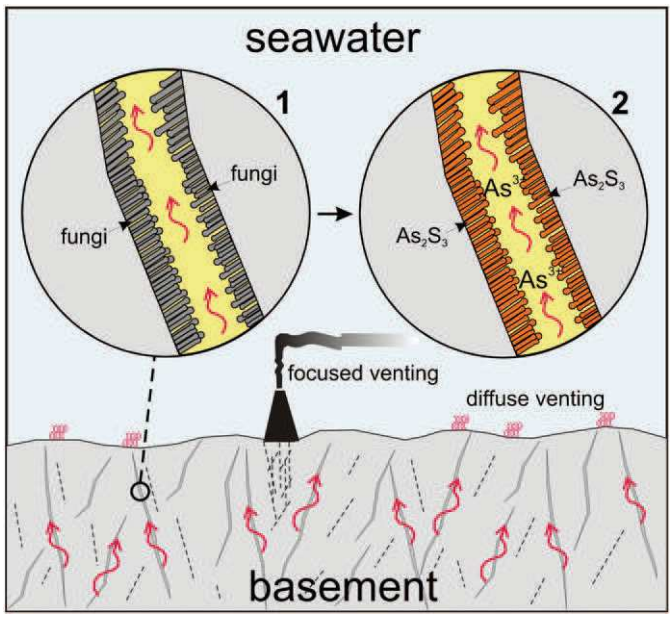

Fig. 8. Schematic scenario of orpiment (As_2S_3) fungal mineralization. A seafloor hydrothermal field with a focused (black smoker) and diffuse venting lies on a permeable basement. Basement permeability is provided by a network of open cracks that are a result of crustal extension. The crack network is the plumbing system of the hydrothermal fluids (red wavy arrows) and a habitat for fungi. Fungi that live in the open cracks (stage 1) are killed by As flux and subsequently mineralized by As_2S_3 (stage 2). For more discussion see the text.

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2. Communications orales

Rédou V., Ciobanu M.C., Dufresne A., Lengellé J., Buée M., Vandenkoornhuyse P., Alain K., Barbier G., Burgaud G. La vie insoupçonnée des grands fonds marins. Journée des doctorants SCIMA, Brest, 26 septembre 2013. Prix de la meilleure communication orale.

Rédou V., Ciobanu M.C., Dufresne A., Lengellé J., Buée M., Vandenkoornhuyse P., Alain K., Barbier G., Burgaud G. 454-pyrosequencing analyses of record-depth subsurface sediments revealed unexpected microeukaryotic communities dominated by fungi. MaCuMBA Project, 2nd Annual Meeting, Station Biologique de Roscoff, France, September 23-27, 2013.

Rédou V., Romac S., Lengellé J., Buée M., Barbier G., de Vargas C., Burgaud G. Fungal communities in European coastal sediments as revealed by 454 pyrosequencing. BioMarKs final meeting, Station Biologique de Roscoff, France, November 19-21, 2013.

3. Communications affichées

Rédou V., Arzur D., Burgaud G., Barbier G. Fungal diversity in deep marine sediments of the Canterbury Basin (NZ). ISME 14, Copenhagen, Denmark 19-24 August 2012.

Rédou V., Ciobanu M.C., Dufresne A., Lengellé J., Buée M., Vandenkoornhuyse P., Alain K., Barbier G., Burgaud G. 454 Pyrosequencing analyses of record-depth subsurface sediments revealed unexpected microeukaryotic communities dominated by Fungi. FEMS 2013, Leipzig, Germany, July 21-25, 2013.

Rédou V., Romac S., Lengellé J., Buée M., Barbier G., De Vargas C., Burgaud G. Fungal communities in European coastal sediments as revealed by 454 pyrosequencing. FEMS 2013, Leipzig, Germany, July 21-25, 2013.

Fungal diversity in deep marine sediments of the Canterbury Basin (NZ)

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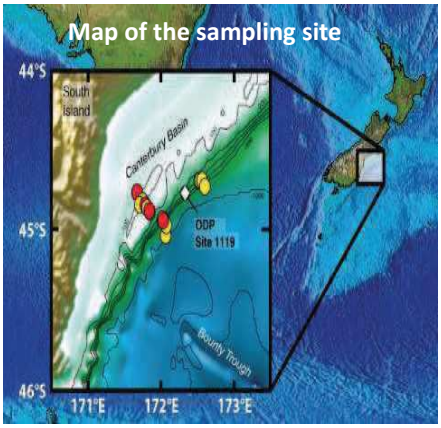
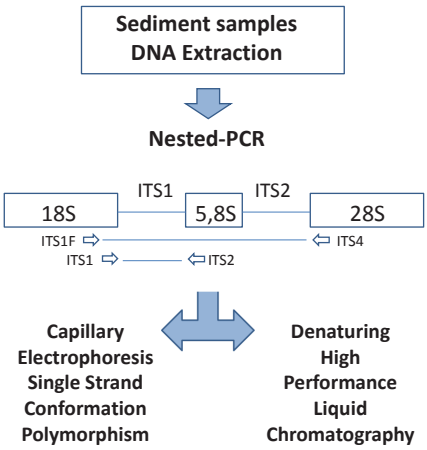
INTRODUCTION

Recent years have seen a growing interest regarding microeukaryotic communities in extreme environments. Recently, fungal signatures were revealed in marine subsurface sediments (40 centimeters below sea floor) of the Central Indian Basin by culture-based and molecular approaches (Singh et al., 2012). These results have confirmed those of a previous study interested in microeukaryotes in marine subsurface sediments of the Peru margin, collected at 37 meters below sea floor. Dominance of fungal communities in microeukaryotes in these extreme ecosystems was demonstrated (Edgcomb et al., 2011).

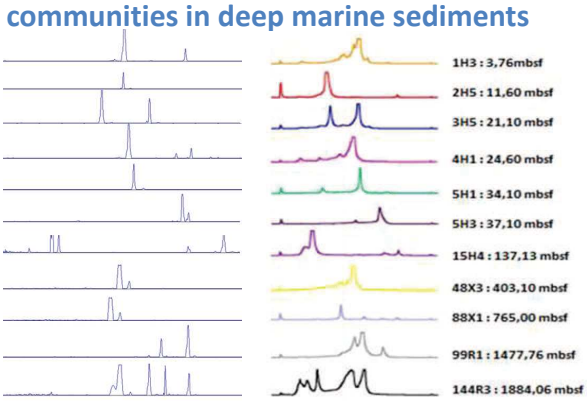
In the present study, we have identified the fungal communities present throughout a sediment core of the Canterbury Basin (New Zealand) from 3,76 to the record depth of 1884 meters below sea floor.

MATERIAL AND METHODS



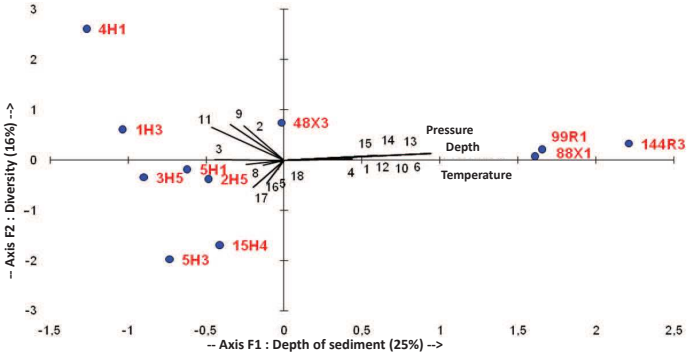


CE-SSCP and DHPLC profiles of fungal communities in deep marine sediments



- PCR/CE-SSCP and PCR/DHPLC gave both correlated results.
- Profiles obtained revealed a complex and heterogeneous molecular diversity of fungi from subsurface to 1884 mbsf.

Principal component analysis of physicochemical data and molecular diversity



- The fungal diversity appears correlated with the depth of the sediment and thus with *in situ* hydrostatic pressure and temperature.

Taxonomic groups

Depth (mbsf)	3,76	11,60	21,10	24,60	34,10	37,10	137,13	403,10	765,00	1477,76	1884,06
Sample	1H3	2H5	3H5	4H1	5H1	5H3	15H4	48X1	88X1	99R1	144R3
	Penicillium citrinum	*Malassezia restricta* *Rhodosporidium kratochvilovae* *Rhodotorula glutinis*	*Rhodotorula glutinis*		*Penicillium citrinum* *Peniophora incamata*	*Malassezia restricta* *Penicillium citrinum*	*Dioszegia crocea* *Peniophora incamata*	*Penicillium citrinum* *Dioszegia crocea*	*Cladosporium sp.* *Malassezia restricta* *Cadophora malorum*	*Glovinomyces cichoracearum*	*Peniophora incamata* *Rhodotorula mucilaginosa*

- Collection of DHPLC peaks allowed direct sequencing and rapid identification of the fungal diversity.
- A low diversity has been demonstrated but fungal comunities in these extreme envionments are thought much more complex.

CONCLUSIONS AND FUTURE DIRECTIONS

- Fingerprinting methods have revealed for the first time a fungal diversity in deep-sea sediments until 1884 meters below sea floor.
- Conventional cloning-sequencing and 454 Tag-pyrosequencing are in progress in order to characterize the whole fungal diversity in deep marine sediments.
- Culture-based methods using high pressure and/or anaerobic equipments mimicking environments will certainly provide useful fungal isolates.

- 14th International Symposium on Microbial Ecology - 19-24 August 2012 - Copenhagen, Denmark -

454 Pyrosequencing analyses of record-depth subsurface sediments revealed unexpected microeukaryotic communities dominated by Fungi

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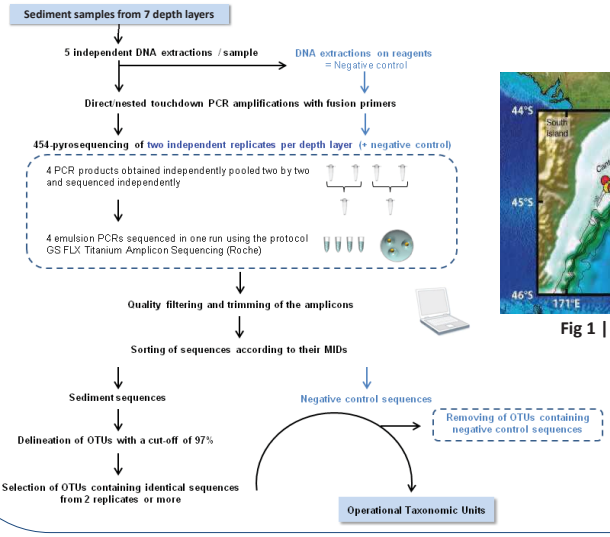
INTRODUCTION

Deep sub-sea-floor biosphere provides a habitat for of widespread microbial life. This subsurface microbiota is diverse and complex, hosting metabolically active communities down to depths of more than one thousand meters below the seafloor. If an extensive background on bacterial and archaeal lineages endemic in deep sea sediment was established, subsurface eukaryotic communities still remained largely underexplored. Ribosomal RNA surveys of superficial marine sediments, *i.e.* first centimeters to first meters, have shown an astonishing diversity of active eukaryotic communities, yet the depth range and diversity of microeukaryotes in the deep subsurface are unknown.

Aim of this study was to investigate vertical distribution of microeukaryotic communities from deep sea sediment collected in the Canterbury basin, off the coast of New Zealand from 1740 mbsf. We developed a tag-pyrosequencing approach targeting the 18S hypervariable regions of small-subunit rRNA gene and fungal ITS1 region.

MATERIALS AND METHODS



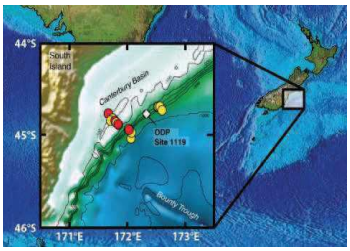


Fig 1 | Map of the sampling site

Vertical distribution of microbial taxa

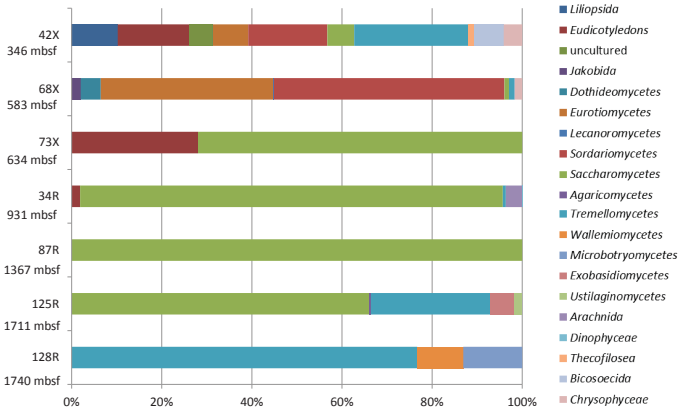


Fig 2 | Class level distribution of eukaryotic 18S rRNA gene-tag sequences based on SILVA111 classification

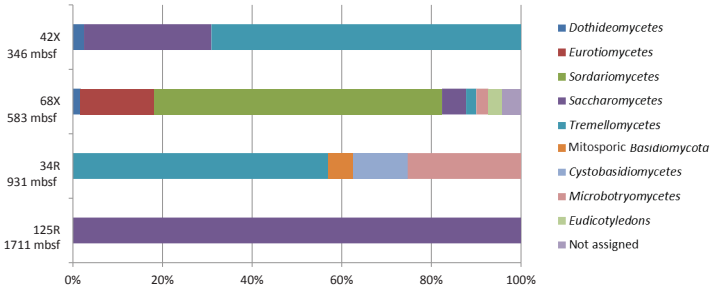


Fig 3 | Class level distribution of fungal ITS1 region-tag sequences based on Genbank classification

Pyrosequencing dataset on extracted DNA

	ITS analysis				18S analysis						
Depth (mbsf)	346	583	931	1711	346	583	634	931	1367	1711	1740
Samples code	42X	68X	34R	125R	42X	68X	73X	34R	87R	125R	128R
Number of reads	4930	3948	1580	3874	6425	5868	7646	4521	2318	2866	2659
Number of retained quality reads	4548	3450	1317	3496	5427	4747	6899	1598	1468	2736	2033
Number of retained OTUs	39	120	40	29	15	26	4	6	2	5	4

Table 1 | Number of sequences and OTUs in the dataset

Diversity and environmental factors

Relationships between eukaryotic 18S rRNA gene-tag sequences and environmental parameters show that the depth is the main factor that define a model of vertical distribution of eukaryotic communities.

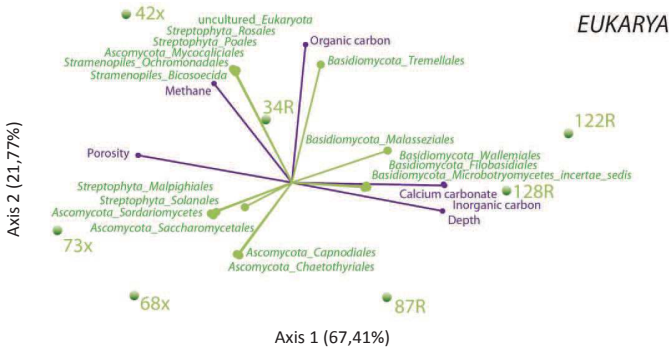


Fig 4 | Principal Component Analyses (PCA) with sediment samples (green dots), microbial taxa (green vectors) and environmental parameters (purple vectors).

CONCLUSIONS AND FUTURE DIRECTIONS

This study specifically deals with fungal communities in the deep sub-sea-floor using two DNA barcodes (18S and ITS of rRNA). We highlighted an unexpected fungal diversity to the record depth of 1740 mbsf using 454-tag-pyrosequencing. The fact that some of the fungal sequences obtained in this work have been previously reported from marine ecosystems supports the hypothesis that fungal communities have an important ecological role in this ecosystem. Future metatranscriptomic and culture-based studies will reveal the active fraction of sub-sea-floor fungal communities and give clues regarding their adaptation to high hydrostatic and lithostatic pressures and their abilities to adapt to oligotrophic conditions.

- FEMS 2013, 5th Congress of European Microbiologists - July 21-25 2013 - Leipzig, Germany -

Fungal communities in European coastal sediments as revealed by 454 pyrosequencing

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INTRODUCTION

Fungi represent a significant part of the microbial diversity on Earth and play important ecological roles in biogeochemical cycles. Fungal communities have been widely studied in terrestrial ecosystems, yet knowledge on deep-sea fungi is still scarce and their ecological role mostly unknown. Marine sediments represent more than two thirds of the Earth's surface and appear as a potential ecological niche for fungi.

OBJECTIVE

Aim of this study was to evaluate the genetic and functional diversity of fungal communities in superficial marine sediments in order to increase our knowledge of fungal communities in deep-sea ecosystems.

MATERIALS AND METHODS

Fig 1 | Map of the sampling site

Nucleic acids extraction

- Co-isolation of RNA and DNA for the recovery of the total nucleic acid content of the sample

Fungal barcode sequencing

- Roche FLX technology

Bioinformatic analysis

- Extraction of flowgram information
- Trimming / Denoising
- Extraction of ITS regions
- Length trimming
- Clustering at 97%
- BLAST on curated NCBI database
- OTU clustering depending on taxonomic assignment

Fig 2 | Comparison between global class level (a) and order level (b) distribution of ITS1 region DNA and cDNA tag sequences

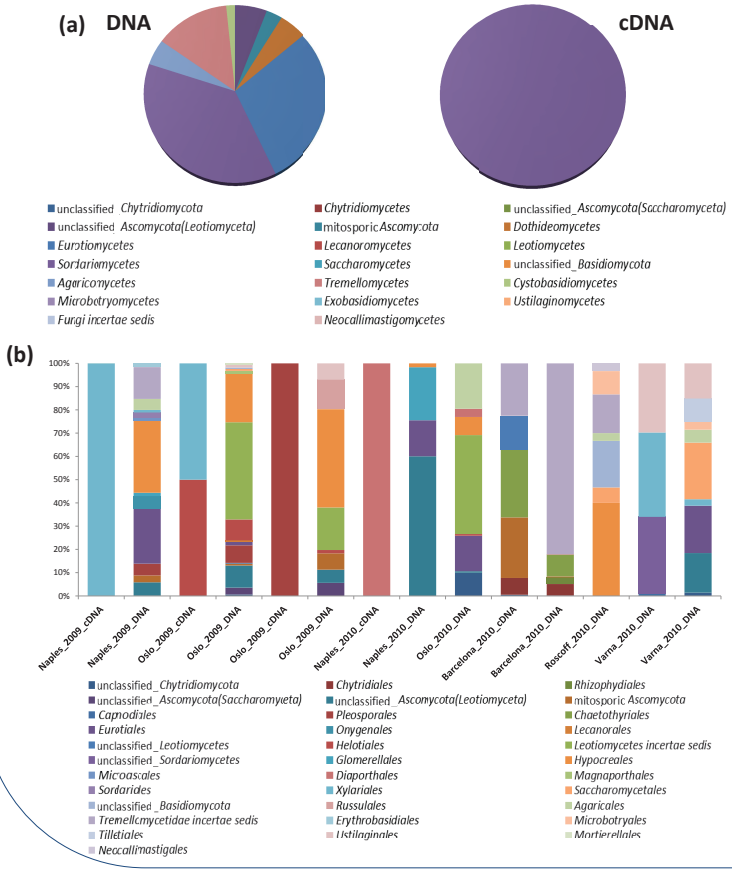

Fig 3 | Taxonomic affiliation of the 20 most abundant fungal OTUs in the dataset

OTU number	Numbers of reads	Gi number	Assignation	Sequence identity	E-value
300	161645	289718876	Uncultured fungus clone	87,3%	$5{,}00^{E}$-24
1831	29426	41411242	*Candida atlantica*	95,1%	$1{,}00^{E}$-89
67	11791	122892467	*Microdochium nivale*	100%	$4{,}00^{E}$-75
2098	5057	289718876	Uncultured fungus clone	86,6%	$1{,}00^{E}$-21
0	2970	122892467	*Microdochium*	100%	$1{,}00^{E}$-75
45	1830	122892467	*Microdochium nivale*	100%	$1{,}00^{E}$-75
586	1755	332107246	Uncultured eukaryote clone	94,9%	$6{,}00^{E}$-16
1497	1490	90398891	*Cryptococcus victoriae*	98,5%	$4{,}00^{E}$-60
3756	1225	37665534	*Cryptococcus* sp.	100%	$1{,}00^{E}$-69
296	1088	332107246	Uncultured eukaryote clone	95%	$4{,}00^{E}$-14
235	980	122892467	*Microdochium nivale*	93,3%	$8{,}00^{E}$-31
423	968	169639290	*Tritirachium* sp.	97,9%	$3{,}00^{E}$-83
2396	960	289718876	Uncultured fungus clone	87,3%	$5{,}00^{E}$-21
1590	934	289718876	Uncultured fungus clone	88,9%	$1{,}00^{E}$-27
3595	807	289718876	Uncultured fungus clone	88,2%	$2{,}22^{E}$-23
2708	794	300676388	*Phomopsis columnaris*	96,7%	$7{,}00^{E}$-64
1820	746	289718876	Uncultured fungus clone	88,9%	$1{,}00^{E}$-27
1500	744	332106336	Uncultured eukaryote clone	88,7%	$7{,}00^{E}$-14
3005	697	312434336	*Ascomycota* sp.	96,6%	$5{,}00^{E}$-52
285	682	193246194	Fungal endophyte isolate	96,3%	$7{,}00^{E}$-64

Only 15% of OTUs were assigned but comprising 45% of the sequences. Among the 30,363 quality ITS reads, taxonomic analyses revealed *Ascomycota* as a dominant phylum (91%) with a majority of *Sordariomycetes, Eurotiomycetes, Leotiomycetes* and *Dothideomycetes. Basidiomycota* appeared less represented (8%) with some *Tremellomycetes, Agaricomycetes* and *Ustilaginomycetes*. Only few sequences were affiliated to *Chytridiomycota* (1%).

CONCLUSIONS AND FUTURE DIRECTIONS

Our results indicate that deep-sea benthic environments harbor a wide diversity of fungi dominated by *Ascomycota*. Diversity and OTU richness suggest a spatial heterogeneity which will be confirmed by multidimensional statistical analyses using taxonomic data and environmental factors. However, the majority of the environmental ITS 454 reads did not cluster with taxonomically known sequences highlighting the main issue to use ITS as a unique barcode to decipher the fungal diversity in uncharacterized exotic ecosystems. Paired-end sequencing strategy targeting both the 18S and ITS rRNA could be considered as an alternative method to assign a greater number of reads and reveal much more ecological information.

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Communautés fongiques des sédiments marins de subsurface : diversité, origine et rôle écologique

Résumé : Au cours des vingt dernières années, les études sur les sédiments marins profonds ont révélé la présence et l'activité de communautés microbiennes inattendues. Il est maintenant formellement établi que la biosphère profonde héberge de nombreux représentants des domaines des *Archaea* et des *Bacteria*. Cependant, les micro-eucaryotes et plus particulièrement les champignons n'ont été que très peu étudiés dans ces écosystèmes singuliers.
Dans ce contexte, des approches moléculaire et culturale ont été utilisées afin de caractériser la diversité des communautés fongiques des sédiments marins profonds en utilisant le bassin de Canterbury comme modèle d'étude. Les résultats principaux obtenus lors de ce travail de thèse sont les suivants : (i) L'approche moléculaire basée sur l'ADN a fourni la preuve directe que les communautés fongiques peuvent persister jusqu'à la profondeur record de 1740 mètres sous la surface du plancher océanique. (ii) Des approches complémentaires ciblant les ARNr et les ARNm ont permis de préciser leur activité métabolique et d'obtenir de premiers indices sur les fonctions de ces champignons à 350m sous la surface du plancher océanique, principalement liées à la croissance, à l'adaptation aux contraintes environnementales in situ et aux interactions entre communautés microbiennes. (iii) L'approche culturale a permis de constituer une collection de culture de 183 isolats fongiques avec des caractéristiques écophysiologiques témoignant leur capacité d'adaptation aux conditions in situ. (iv) Le potentiel biotechnologique des isolats obtenus a été estimé via la recherche de gènes impliqués dans la synthèse de métabolites secondaires et a permis de positionner cette collection d'organismes originaux comme une ressource d'intérêt biotechnologique potentiel.
Ce travail qui témoigne de la persistance et de l'activité des communautés fongiques dans les sédiments marins profonds élargit notre vision de la diversité microbienne dans ces milieux et soulève des hypothèses sur le rôle écologique des champignons au sein de la biosphère profonde.

Mots clés : Communautés fongiques, *Archaea* et *Bacteria*, bassin de Canterbury, sédiments marins profonds.

Fungal communities in deep subsurface sediments: diversity, origin and ecological role

Abstract : Over the past two decades, investigations on deep marine sediments have revealed the occurrence and activity of unexpected microbial communities. Many representatives of *Archaea* and *Bacteria* were reported but micro-eukaryotes and especially fungal communities are still poorly studied in this ecosystem.
In this underexplored context, molecular- and culture-based approaches were used to characterize the diversity of fungal communities in deep subsurface sediments using the Canterbury Basin as a model system. The main results of this work are: (i) The molecular DNA-based approach provided direct evidence that the fungal communities persist until the record depth of 1,740 meters below sea floor. (ii) Supplementary approaches targeting rRNA and mRNA revealed their metabolic activity and highlighted first hints into the fungal functions at 350 meters below sea floor, mainly related to growth, adaptation to in situ environmental constraints and microbial interactions. (iii) The culturebased approach allowed establishing a culture collection of 183 fungal isolates with ecophysiological characteristics indicating their ability to adapt to in situ conditions. (iv) This culture collection seems to represent a reservoir of secondary metabolites as many genes involved in secondary metabolites pathways were revealed. The fungal collection established may be considered as an untapped resource to explore for biotechnological applications.
This work demonstrating the persistence and activity of fungal communities in deep subsurface sediments (i) broadens our view of microbial diversity in these environments and (ii) raises hypotheses about the ecological roles of fungi in the deep biosphere.

Keywords : Fungal communities, *Archaea* and *Bacteria*, Canterbury basin, deep subsurface sediments.